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Our results suggest that estrogen receptor mediated signal transduction may be quite complex in human breast cancer. We have identified the frequent expression of several alternatively spliced variants of the classical ER-α as well as a second novel ER gene (ER-β) , at least, at the mRNA level, in both normal and neoplastic human breast tissues. Further we have both published and preliminary data showing alterations in the relative expression of several of these ER-like molecules during human breast tumorigenesis and breast cancer progression. Our data suggest a possible role(s) of these ER-like molecules in altered ER signal transduction during breast tumorigenesis and progression.

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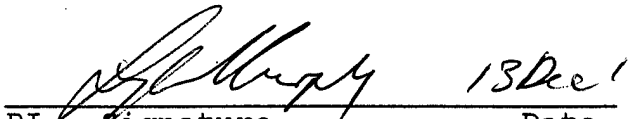

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## 4. TABLE OF CONTENTS.

## 5. INTRODUCTION:

**The goal of this research is to address the role of variant/abnormal estrogen receptor (ER) expression in the progression of human breast cancers from hormone dependence to independence**. The progression of breast cancer from hormone dependence to independence is a clinically significant problem since it limits the effectiveness of the relatively non-toxic hormonal therapies such as antiestrogens and progestins (1). The hormone-dependent phenotype is characterized by the presence of ER in the breast tumor, but only 50% of receptor positive breast tumors respond to endocrine therapies and of those which initially respond a significant proportion will eventually develop resistance to these therapies. Furthermore, the development of resistance to endocrine therapy occurs despite the continued expression of ER in the tumor, in at least 50% of cases. It is the molecular mechanisms of this form of resistance i.e. the steroid receptor positive/hormone resistant human breast tumors, that this research proposal addresses. Elucidation of these mechanisms will provide information necessary either to prevent the occurrence of hormone resistance, reverse it or develop new treatments for the resistant tumors. As well, novel treatment response markers in human breast tumors are likely to be identified. Although multiple mechanisms are likely to be involved in hormone resistance and progression to hormone independence (1) in human breast cancer, this grant proposal focuses on one possible mechanism: the involvement of variant and/or abnormal forms of ER.

The hypothesis to be tested is that the expression and/or altered expression of variant/abnormal ER in human breast cancer is one mechanism associated with the development of endocrine resistance and the progression from hormone dependence to independence.

The specific aims to address this hypothesis are:

1. To systematically investigate alterations that occur in the ER mRNA in human breast cancers.
2. To characterize structurally and functionally those abnormal ER mRNAs occurring most frequently and determine their involvement in the development of hormone independence and progression in HBC.
3. To develop specific tools to investigate the expression of the corresponding proteins that may be translated from altered or variant ER mRNAs.

4. To assess the biological significance of ER variants in human breast cancer by determining the relationship between the level of expression of ER variants in human breast cancer biopsies and the expression of the normal ER, known estrogen responsive genes, histopathological parameters and other known prognostic factors .

**6. BODY:**

**Task 1.** To systematically investigate alterations that occur in the ER mRNA in human breast cancers. We have systematically investigated alterations that occur in the estrogen receptor (ER) mRNA in human breast tumors. Several manuscripts and abstracts reporting these studies have been published (see references 2-5). During the past year of this grant the identification of another estrogen receptor gene, estrogen receptor-β was reported (6). The ER-β protein has similarities to the classical ER referred to as ER-α, in terms of structure and function. Both these proteins have a high degree of conservation of the DNA and ligand binding domains, while the A/B, hinge and F domains are not conserved. The tissue specific expression of ER-α and ER-β although not identical appears to overlap in some cases (6). The expression of ER-β in normal or neoplastic mammary tissue was not reported, and it became extremely important to determine if ER-β (another possible ER isoform/variant) is expressed in breast cancers and could therefore potentially contribute to estrogen signal transduction in this tissue. We investigated this possibility and were the first to publish the detection of ER-β mRNA in several human breast tumor biospy samples and several human breast epithelial cell lines using RT-PCR analysis (7). The expression of ER-β was not correlated with that of ER-α and both ER positive and negative cell lines as determined by ligand binding assay, expressed ER-β mRNA. However, some breast tumors and some cell lines coexpress ER-β and ER-α mRNA. Our data support a role for ER-β in human breast cancer. Further, our preliminary data show the presence of ER-β mRNA in normal human breast tissue (8), and suggest that the level of this gene is differentially expressed between normal breast tissue and human breast tumors (Fig 1, appendix 5). Indeed there is a possibility that the ER-β is inversely regulated compared to ER-α (Fig. 1, appendix 5) in these two tissues. We have also demonstrated the presence of variant forms of ER-β mRNA in several human and mouse tissues (8), although species specific differences were found to occur.

**Task 2.** To develop quantitative RT-PCR assays for measuring selected ER mRNA variants in RNA isolated from microdissected regions of human breast tumors. Several different assays were developed, each with different advantages and limitations. These results have been previously published (4, 9-13). The type of assay used, was dictated by the the question asked and practical issues. These assays are being used to assess the biological significance of ER-α variants in human breast cancer under Task 5. However, in order to compare some of our current data with that obtained previously (14), it was necessary to validate the comparsion of our RT-PCR based assays with RNase protection assays. The results of this validation are presented in the accompanying manuscript (15) which will be submitted for publication shortly. Due to our demonstration of the expression of ER-β mRNA in human breast tumors and the possible differential regulation of ER-β and ER-α we have also developed a multiplex assay to determine the relative expression of these two ER genes in human breast tissues (Fig 1C, appendix 5).

**Task 3.** To characterize structurally and functionally those variant ER mRNAs occurring most frequently in human breast cancer. Eukaryotic expression vectors for several of the variant and mutant ER-α mRNAs have been constructed and used to determine the ability to bind ligand, transcriptional activity alone or alter transcriptional activity of the wild type ER-α (5). Previously we had developed a model of estrogen independence in human breast cancer cells (16). We have now identified a marked upregulation of the relative expression of an exon 3+4 deleted ER-α variant mRNA in the estrogen independent cells (T5-PRF) compared to the T5, estrogen responsive parent cells (17). Furthermore, the estrogen independent phenotype of the T5-PRF cells was associated with a significant increase in the basal, estrogen independent activity of the wild type ER-α, as determined by transient transfection of an ERE-tk-CAT reporter gene (Fig 2, appendix 5), and the measurement of endogenous PR expression (Fig 3, appendix 5). The exon 3+4 deleted ER-α mRNA is inframe and predicted to encode a 49 kDa protein missing the second zinc finger of the DNA binding domain, the nuclear localization domain and part of the hormone binding and E domain. An expression vector containing the exon 3+4 deleted ER-α cDNA was constructed. The *in vitro* transcribed/translated protein does not bind estradiol and does not interact with an ERE by gel shift assay. But, when the expression vector was transfected into the estrogen responsive parent T5 cells, a dose-dependent

increase in basal, ligand independent activity of the endogenous wild type ER-α occurred (Fig 4, appendix 5). Our preliminary data suggest that when transfected alone into MDAMB231 (ER-α negative human breast cancer) and MCF10A1 (ER-α negative nontumorigenic, immortalized human breast epithelial) cells with an ERE-tk-CAT reporter the exon 3+4 deleted ER-α has no detectable transcriptional activity. But when reconstituted with wild type ER-α using a constant amount of HEGO (wild type ER-α expression vector) and increasing exon 3+4 deletion ER-α plasmid, increased ligand independent (control) ERE-tk-CAT activity is seen in both MCF10A1 and MDAMB231 cells (Fig 5, appendix 5). As well an enhancement of estradiol induced ERE-tk-CAT activity is observed when both wild type ER-α and exon 3+4 deletion ER-α are expressed together.

We are also constructing eukaryotic expression vectors for both human and mouse ER-β and their frequently expressed variants.

**Task 4.** To develop specific tools (antibodies) to investigate the expression of the corresponding proteins that may be translated from variant ER-α mRNAs.

A synthetic peptide containing the novel 6 amino acids present in the predicted clone 4 ER-α protein was conjugated to KLH (Chiron Inc) and this antigen was used to raise antibodies to the clone 4 ER-α variant protein in rabbits (National Biological Labs). The antisera have been prescreened against the synthetic peptide, and the most promising antisera were used to screen Cos-1 and MCF-7 cells stably transfected with the clone 4 variant ER-α transgene. Our preliminary data show that the antiserum (Clone 4 Ab) can detect a 24 kDa protein present in transgene transfected Cos-1 and MCF 7 (Fig 6, appendix 5) but not in vector alone transfected cells. Further, a similar sized protein is also detected with H226 antibodies (1D5, which detects an N-terminal epitope of the wild type ER-α which is predicted to be present in the clone 4 variant ER-α protein). These data are encouraging and consisent with the antiserum containing specific antibodies to the clone 4 ER-α protein. At 1/100 dilution the antiserum appears to detect other larger proteins on Western blots. Serum dilutions and excess peptide immunoneutralization assays are being undertaken to further characterize the antiserum. Immunoglobulin isolation and affinity purification will also be undertaken. The ability of the antibodies to immunoprecipitate clone 4 ER-α protein will also be determined. Following the satisfactory completion of this characterization we will be able to begin the

analysis of human breast tumor samples to determine the presence of clone 4 ER-α-like epitopes and proteins *in vivo*.

Previously, we had analyzed a group of human breast tumors immunohistochemically for expression of ER-α using antibodies that recognize either an N-terminally localized epitope in the wild-type ER-α protein (expected to be present in many ER-α variants), or a C-terminally localized epitope in the wild type ER-α protein (not expected to be present in many ER-α variants). In many tumors the immunohistochemical results using each antibody showed good concordance, in some tumors the results were discordant, with the signal tending to be higher more often with the N-terminal antibody (18). Since many of the proteins predicted from variant ER-α mRNAs would be truncated at the C-terminus and not contain the epitope recognized by the C-terminal antibody, one interpretation of these data was that truncated variant ER-α proteins are more highly expressed in the discordant group of tumors. This hypothesis was tested by investigating the pattern and relative expression of variant ER-α mRNAs in the discordant and concordant groups of breast tumor (19) . We used our previously developed assays (4,11) to determine the relative pattern of expression of exon deleted ER-α mRNAs and to measure the relative expression of the clone 4 truncated ER-α mRNA in these two groups of breast tumors. Several ER-α variant mRNAs which encode putative truncated ERs recognized only by an N-terminal targeted antibody were preferentially and relatively more highly expressed in the discordant group of tumors. These ER-α variants were: the clone 4 truncated ER-α mRNA, an exon 2, 3 plus 7 deleted ER-α mRNA, an exon 2, 3 and 4 deleted ER-α mRNA and a variant deleted from within exon 3 to within exon 7. The data suggest that the ER-α variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER-α measured by immunodetection methodologies. Further, the data are consistent with the ability of some ER variant mRNAs to be stably translated *in vivo* .

**Task 5.** <u>To assess the biological significance of ER variants in human breast cancer.</u> Available studies provide evidence for an extensive and complex pattern of alternative splicing associated with the ER-α gene, which seems to be altered during breast tumorigenesis (4, 9,11, 20). It has been suggested that the complex pattern of exon deleted ER-α variant mRNA expression is specific for the ER-α, since similar variants for the glucocorticoid receptor and the retinoic acid receptors-α and γ have not been found in breast tumor tissues. We also investigated the pattern of exon deleted variant mRNA expression in breast

tumors using a long range RT-PCR approach (4) for progesterone receptor (PR), the glucocorticoid receptor (GR) and the vitamin D3 receptor (VR). Our data (21) demonstrate that little, if any, deleted mRNAs for GR and VR were detected in the breast tumors examined. However, in these same breast tumors, several exon deleted variant mRNA species for both ER-α and PR were abundantly expressed (21). These data suggest that the mechanisms generating alternatively spliced forms of both ER-α and PR are unlikely to be due to a generalized deregulation and/or alteration of splicing processes within breast tumors. They also suggest that the mechanism(s) is specific for the sex steroid hormone receptor genes and the alterations seen in breast tumors may have a role in altered actions of estrogens and progestins in human breast tumorigenesis.

We have previously shown that alteration of expression of some variant ER-α mRNAs was found to occur during both breast tumorigenesis (9,11) and breast cancer progression (4, 14). With regard to the latter, we showed that the expression of the truncated, clone 4 variant ER-α mRNA (14) relative to wild type ER-α mRNA was significantly increased in a group of primary breast tumors with poor prognostic features compared to a group of primary breast tumors with good prognostic features (14). "Poor" prognostic features included the presence of lymph node metastases at the time of surgery, large tumor size, lack of PR expression and high proliferative index, while "good" prognostic features were lack of nodal involvement, small tumor size, PR positivity and low proliferative index. This suggested that altered ER-α variant expression may be a marker of a more aggressive phenotype. We have investigated this possibility further by comparing the over-all pattern of deleted ER-α variant expression between matched primary tumors and their concurrent lymph node metastases, as well we have compared the relative level of expression of several individual ER-α variant mRNAs in these matched tumor samples. Our data (15) suggest that both the pattern of ER-α variant expression and the relative level of expression of three individual ER-α variants are conserved in primary breast tumors and their matched, concurrent lymph node metastases. These findings are not inconsistent with our previously published data in which the relative expression of at least one ER-α variant was significiantly increased in primary tumors with poor prognostic characteristics, which included having concurrent lymph node metastases, as compared to primary tumors without concurrent lymph node metastases (14). The primary tumors in our current study by definition fall into

the previously described poor prognostic group since they are all primary breast tumors with concurrent lymph node metastases.

## 7. CONCLUSIONS.

Our results suggest that estrogen receptor mediated signal transduction may be quite complex in human breast cancer. We have identified the frequent expression of several alternatively spliced variants of the classical ER-$\alpha$ as well as a second novel ER gene (ER-$\beta$), at least, at the mRNA level, in both normal and neoplastic human breast tissues. Further we have both published and preliminary data showing alterations in the relative expression of several of these ER-like molecules during human breast tumorigenesis and breast cancer progression. Our data suggest a possible role(s) of these ER-like molecules in altered ER signal transduction during breast tumorigenesis and progression.

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# APPENDIX 1.

*Breast Cancer Research and Treatment* **44**: 145–151, 1997.


*Report*

# A point mutation in the human estrogen receptor gene is associated with the expression of an abnormal estrogen receptor mRNA containing a 69 novel nucleotide insertion

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*Key words:* estrogen receptor, mutation, breast cancer, gene expression

**Summary**

A novel ER-like mRNA containing a 69 nucleotide insertion precisely between exon 5 and 6 sequences was previously identified in human breast cancer biopsy samples. Data are presented which suggest that the 69 nucleotide sequence is normally present in intron 5 of the human estrogen receptor gene. The region corresponding to and surrounding this 69 nucleotide sequence was cloned and the nucleotide sequence determined. Cloning and sequencing of the corresponding region in genomic DNA isolated from a breast tumor expressing the 69 nucleotide inserted ER mRNA, revealed an A—> G point mutation immediately 3′ to the 69 nucleotide sequence. This point mutation resulted in the generation of a consensus splice donor site. A consensus splice acceptor site sequence is normally present immediately 5′ to the 69 nucleotide sequence. These data are consistent with the 69 nucleotide sequence being recognized as an exon by the splicing machinery, and resulting in processing of a mature ER mRNA containing the 69 nucleotide insert.

## Introduction

We have previously identified in approximately 9% of human breast tumors estrogen receptor (ER)-like mRNAs which contain inserted sequences [1]. Two types of inserted sequences were identified: one in which complete duplications of normal ER exons were found and one in which 69 novel nucleotides had been inserted between the exons 5 and 6 sequences of the normal ER mRNA. Other altered ER-like mRNAs have also been found in human breast tumors [2]. However, these were mostly truncated ER-like mRNAs [3], or exon deleted ER-like mRNA [4, 5], both of which were most likely generated by some alternative splicing mechanism [6]. It was difficult, however, to suggest an alternative splicing mechanism for either the exon duplicated transcripts or the 69 nucleotide inserted transcript. More likely these transcripts were generated from a mutated ER allele present in some human breast tumors. In this study we present evidence which supports the presence of a mutated ER allele in a breast tumor from which a 69 nucleotide inserted ER-like mRNA was identified and cloned.

## Materials and methods

*RNA isolation:* Total RNA was isolated from human breast cancer biopsy samples as previously de-

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scribed [7, 8]. The integrity of the RNA was confirmed by denaturing gel electrophoresis [8].

*DNA isolation and Southern blot analysis:* Southern blot analysis and isolation of genomic DNA from human tumors was as previously described [7]. DNA from bacteriophage clones containing genomic fragments of the human estrogen receptor gene (GHER: gift from Dr P. Chambon) [9] was isolated using a plate lysis method [10]. Southern blotting of bacteriophage plaque lifts was carried out according to standard methods [11].

*PCR:* The thermal profile used was 30 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. The reaction volume was 50 µl containing 0.01 M Tris-HCl (pH 8.3), 0.05 M KCl, 2.5 mM $MgCl_2$, 0.2 mg/ml gelatin, 0.2 mM dNTPs, 1 µl of each primer (200 ng/µl), and 1 unit of Taq DNA Polymerase (Gibco/BRL). 20 µl of the PCR reactions were electrophoresed in agarose gels (1–2% depending on expected sizes of products) and visualized by ethidium bromide staining. The primer sequences used are as follows:

Set A:

Upper (sense) 5'-TTT GCT CCT AAC TTG CTC TTG – 3' (priming site in exon 5).

Lower (antisense) 5' – CGT AAC TGG AGG AAG TGG – 3' (priming site in novel 69 nucleotides).

Set B:

Upper (sense) 5' – TGC CAG TAG CAA CCT CCA CTT – 3' (priming site in novel 69 nucleotides).

Lower (antisense) 5' – CGG AAC CGA GAT GAT GTA GCC – 3' (priming site in exon 6).

Set C:

Upper (sense) 5' – TGC CAG TAG CAA CCT CCA CTT – 3' (priming site in novel 69 nucleotides).

Lower (antisense) 5' – CGT AAC TGG AGG AAG TGG – 3' (priming site in novel 69 nucleotides).

Set D:

Upper (sense) 5' – CCC AGT CTC AGG TAG GTC TTT – 3' (priming site in intron 5, 5' to novel 69 nucleotides).

Lower (antisense) 5' – GAG TTG GGA AAG CAT AGA GTG – 3' (priming site in intron 5, 3' to novel 69 nucleotides).

*Preparation of radiolabelled probes:* The human ER cDNA (OR-8) [12] was labelled with $^{32}P$ by nick-translation as described previously [7]. A specific probe for the novel 69 nucleotide sequence was prepared by PCR amplification using primer set C (see above). This primer set generates a 64 bp PCR product which was separated from free nucleotides and primers by low melting point agarose gel electrophoresis (NuSieve GTG Agarose; FMC, Rockland, ME). This product was labelled with $^{32}P$-dCTP (0.33 µM final concentration) using 1 PCR cycle under the conditions described above except that the other nucleotides were at a final concentration of 0.33 µM each. The labelled fragment was separated from unincorporated radionucleotides on a Sephadex G-10 column.

*Long PCR:* This was accomplished using a Perkin Elmer GeneAmp XL Kit (Roche Molecular Systems Inc., Branchburg, NJ) according to the manufacturer's instructions.

*Cloning and sequencing:* PCR products were isolated from low melting point agarose gels (NuSieve GTG, FMC Bioproducts, Rockland, ME) and ligated into the TA cloning vector, pCR ™II using the TA Cloning ™Kit (Invitrogen, San Diego, CA). The inserts were sequenced using the T, Sequencing Kit (Pharmacia, Baie d'Urfe, Quebec). The region surrounding the 69 nucleotide sequence present in DNA obtained from a tumor expressing the abnormal ER-like transcript was cloned using primer set D.

## Results

We had previously identified 3 human breast tumors which expressed a novel ER mRNA which contained a 69 nucleotide insert precisely between exons 5 and 6 of the wild-type ER mRNA [1]. Due to the precise insertion it was hypothesized that the 69 nucleotide sequence was normally present in in-

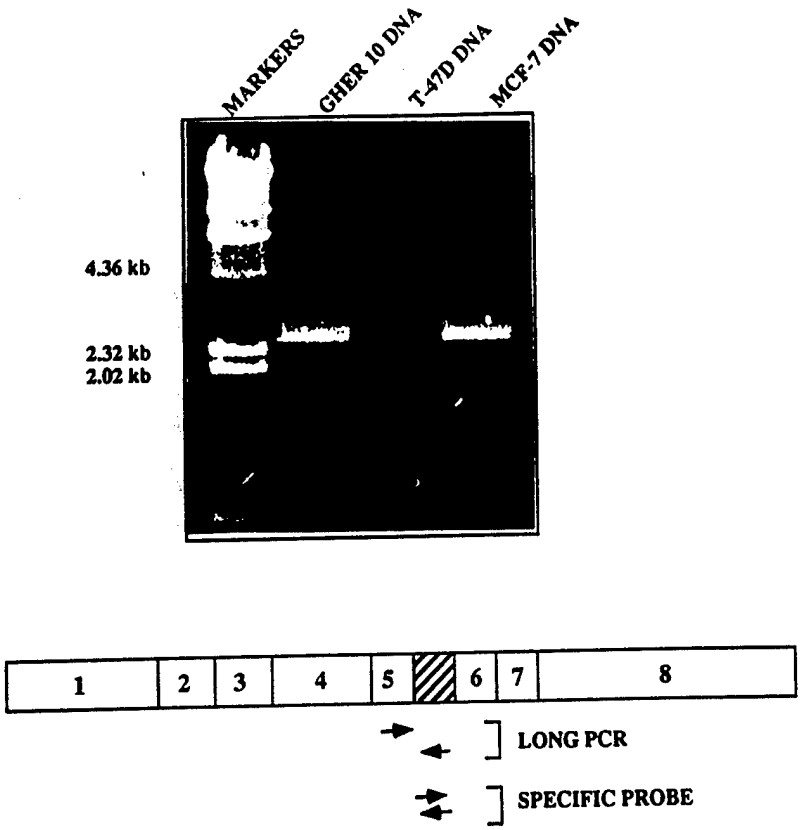



*Figure 1.* The top panel shows the results of long PCR using primer set A of DNA isolated from GHER 10 bacteriophage containing part of the human estrogen receptor gene [9], and genomic DNA isolated from T47D and MCF-7 human breast cancer cells. Primer set A produced a similar approximately 2.5 kbp fragment as visualized by ethidium bromide staining, from all three DNA samples. Markers are Hind III restriction fragments of lambda phage DNA. The bottom panel shows a schematic representation of the abnormal ER-like transcript with the novel 69 nucleotide insertion (cross-hatched box). The arrows show the approximate positions of primer set A (long PCR) and primer set C (specific probe).

tron 5 of the human estrogen receptor gene and that a mutation in the ER gene of those tumors expressing the abnormal ER mRNA resulted in the 69 nucleotide sequence being recognized as an exon and thus being processed into the mature mRNA. This hypothesis predicts that the 69 nucleotide sequence is present in intron 5 of the normal human ER gene. To address this hypothesis two experiments were undertaken.

Firstly, genomic DNA from T-47D and MCF-7 human breast cancer cells, which do not express the 69 nucleotide inserted abnormal ER mRNA, was isolated and subjected to PCR analysis using specific primers for the 69 nucleotide sequence matched with primers either to sequences in wild-type exon 5 (primer set A) or wild-type exon 6 (primer set B). No PCR products were obtained with primer set B (upper primer specific for the novel 69 nucleotide sequence and lower primer specific for exon 6). However, with primer set A (upper specific for exon 5 and lower specific for the 69 nucleotide sequence), a PCR product of approximately 2.5 kbp was obtained in DNA isolated from both these cell lines (Figure 1).

Secondly, several genomic clones spanning the regions around exon 5 and exon 6 of the human ER (gift from Dr P. Chambon) [9] were subjected to Southern blotting of bacteriophage plaques. Duplicate lifts were made. One filter was hybridized with radiolabelled hER cDNA while the duplicate lift

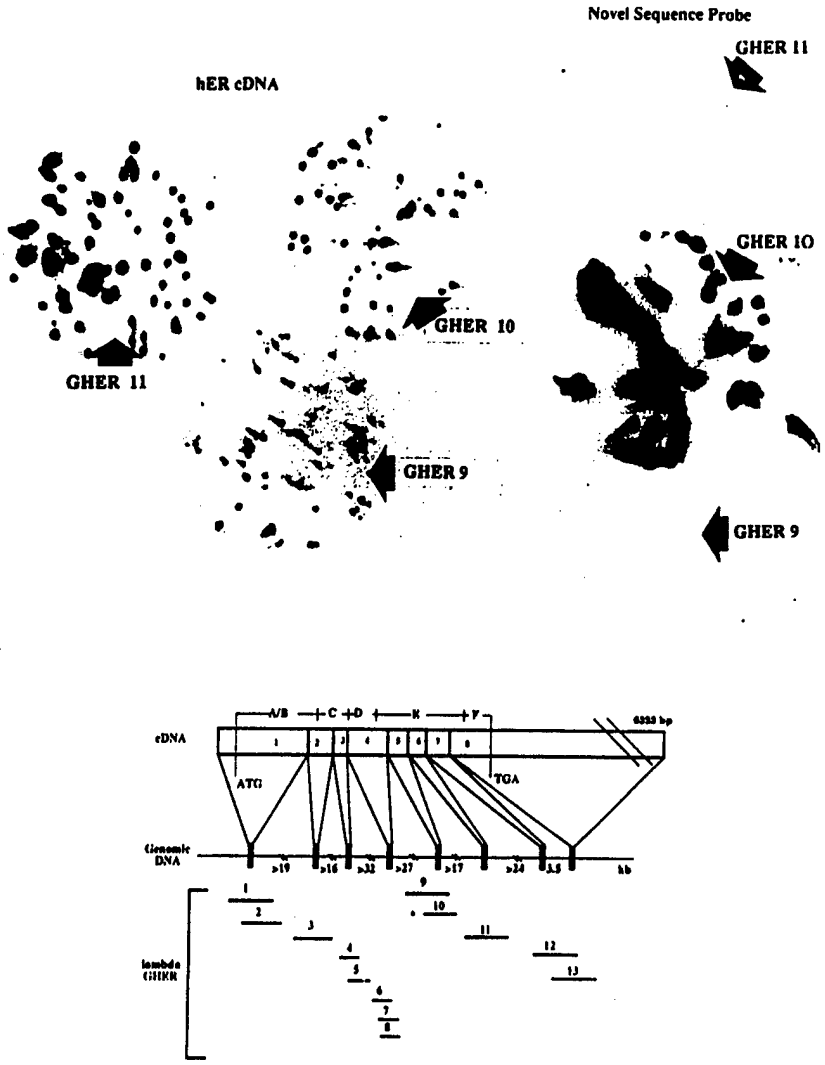



*Figure 2.* Top panels: Bacteriophage containing genomic clones of the regions around exons 5 and 6 of the human estrogen receptor (GHER 9, GHER 10, GHER 11) [9] were subjected to plaque lifts and Southern blotting, using either a human estrogen receptor cDNA (OR-8) [12] or a probe specific for the novel 69 nucleotide insertion (novel sequence probe, see Figure 1). The bottom panel shows a schematic representation of the human estrogen receptor cDNA, human estrogen receptor gene, and the bacteriophage clones containing various regions of the human estrogen receptor gene [9].

was hybridized with a radiolabelled 64 bp probe specific for the novel 69 nucleotide sequence (primer set C). Figure 2 shows the results of this experiment. Human genomic ER clones GHER 9, 10, and 11 all hybridized with the hER cDNA, but only GHER 10 hybridized with the 64 bp probe specific for the novel 69 nucleotide inserted sequence. When DNA isolated from GHER 10 was subjected to long PCR using the primer set A (upper specific for exon 5 and lower specific for the novel 69 sequence), a PCR product of approximately 2.5 kbp was obtained which appeared to be identical in size to that obtained from DNA isolated from the breast cancer cell lines (Figure 1).

Digestion of DNA isolated from GHER 10 with Eco RI yielded four bands. Southern blotting and hybridization with the 64 bp radiolabelled probe specific for the novel 69 nucleotide sequence identi-

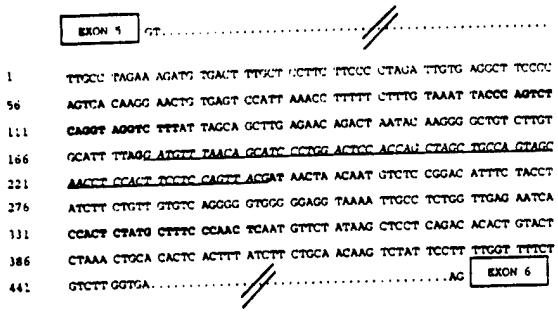



*Figure 3.* Wild-type nucleotide sequence of the region surrounding the 69 nucleotide sequence, which was subcloned from DNA isolated from GHER 10 bacteriophage. The 69 nucleotide sequence is in italics and underlined. The AG immediately upstream of the 69 sequence is bold-faced, as is the AT immediately downstream of the 69 nucleotide sequence. The two bold-faced 21 nucleotide sequences upstream and downstream of the 69 nucleotide sequence are the sequences used to generate PCR primers (primer set D) which were then used to clone the corresponding region from DNA of a breast tumor expressing the 69 nucleotide inserted ER mRNA.

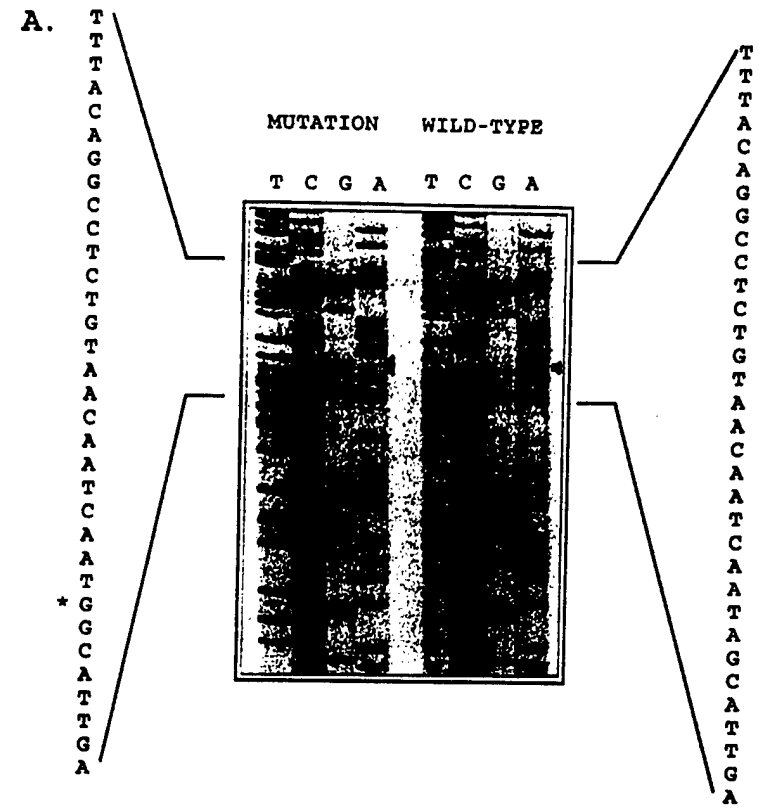
fied only one band of approximately 3 kbp (data not shown). This band was subcloned into the plasmid Bluescript SK-(Stratagene, La Jolla, CA) and primers specific (sense and antisense) for the novel 69 nucleotide sequence were used individually as sequencing primers. The nucleotide sequence obtained from this analysis confirmed the presence of the novel 69 nucleotide sequence within the clone. As well, the sequence of approximately 170 nucleotides either side of the novel 69 nucleotide sequence was also obtained (Figure 3). As shown in Figure 3, the sequence immediately 5′ to that of the novel 69 nucleotide sequence, is AG. This is a potential splice acceptor site to partner the splice donor site at the end of exon 5. The sequence immediately 3′ of the novel 69 nucleotide sequence is AT. We reasoned that the simplest explanation for the 69 nucleotides being recognized as an exon in the tumor cells expressing the abnormal ER-like transcript, would be a mutation which generated a new splice donor site at the end of the 69 nucleotides which could partner the splice acceptor site preceding exon 6.

Therefore, DNA was isolated from the human breast tumor biopsy sample originally found to express the 69 nucleotide inserted ER-like mRNA (see Figures 2 and 3 in reference 1). The region surrounding the novel 69 nucleotide sequence was selected and PCR amplified using primers designed from the known normal sequence around this area (Figure 3, primer set D). These primers amplified the expected 252 bp fragment in DNA isolated from non-expressing tumors as well as from the abnormal ER-like mRNA expressing tumor. The 252 bp fragment from the expressing tumor was subcloned and sequenced. Two independent PCRs led to clones some of which when sequenced contained an A——> G mutation immediately 3′ to the novel 69 nucleotide sequence (Figure 4). This mutation generates a new splice donor site to partner the splice acceptor site preceding exon 6. Such a mutation would be consistent with the novel 69 nucleotide sequence being recognized as an exon and being processed into the mature ER-like mRNA. It should be noted that clones containing the wild-type sequences were also obtained.

## Discussion

Several variant ER-like transcripts have been characterized in human breast cancers [2]. They fall into two main categories: the precise exon deleted transcripts [4, 5] and the truncated transcripts [3, 7], both of which are likely to be generated by an alternative splicing mechanism [6]. More recently we have identified an ER-like transcript which was larger than the wild-type ER mRNA, and indeed cloning and sequencing of this transcript identified a precise insertion of 69 nucleotides between exon 5 and exon 6 sequences [1]. It seemed more likely that this novel transcript was generated from a mutant ER gene, rather than some alternative splicing mechanism. The data presented in this manuscript identify a point mutation in the ER gene present in DNA isolated from the tumor originally found to be expressing high levels of the novel 69 nucleotide inserted ER-like mRNA [1].

The 69 nucleotide sequence was found to be present in DNA obtained from cells containing only the wild-type estrogen receptor gene, at least as defined by the lack of expression of any detectable abnormal inserted ER-like mRNA. The sequence was further mapped to intron 5 of the human estrogen




B.

AT

TAGCA GCTTG AGAAC AGACT AATAC AAGGG GCTGT CTTGT

GCATT TTAG*G ATGTT TAACA GCATC CCTGG ACTCC ACCAG*

*CTAGC TGCCA GTAGC AACCT CCACT TCCTC CAGTT ACG*GT

AACTA ACAAT GTCTC CGGAC ATTTC TACCT ATCTT CT

*Figure 4.* Nucleotide sequence of wild-type and mutant clones obtained from the PCR analysis (primer set D) of DNA from a breast tumor expressing the 69 nucleotide inserted ER mRNA. The arrowheads on the sequencing gel identify the relevant nucleotides in each clone. The asterisk identifies the G point mutation in the mutant clone. Panel A shows representative sequencing gels of both wild-type and mutant clones, and panel B shows the mutant nucleotide sequence, with the 69 nucleotides in italics and underlined and the G mutation bold-faced.

receptor gene [9]. Sequencing of the region surrounding the 69 nucleotide sequence in the wild-type gene identified a consensus splice acceptor site immediately 5′ to the 69 bp sequence, but not a donor splice site immediately 3′ to the 69 bp sequence. When the same region was characterized in DNA isolated from the tumor expressing the abnormal transcript, an A—> G transition was found immedi-

ately 3′ to the 69 nucleotide sequence which generated a new consensus splice donor site. Indeed, the presence of the 69 nucleotide inserted ER-like mRNA in this tumor is consistent with the 69 nucleotides being surrounded with appropriate splicing signals and being recognized as an exon and therefore processed into mature mRNA.

Although wild-type clones were also obtained from the DNA of the tumor expressing the abnormal ER-like mRNA, the A—> G mutation is unlikely to be a PCR induced error, since the identical mutation was obtained from clones generated from two independent PCR reactions. The mixture of wild-type and mutant is more likely to reflect the presence of both the wild type allele and the mutant allele in the tumor sample. Further, such data are consistent with the original RT-PCR data in which both the wild-type RT-PCR product and the abnormal RT-PCR product were observed in the same RNA extract [1]. It is unclear whether this represents heterozygosity for the mutant allele or alternatively heterogeneous cell populations within the tumor sample. Moreover, we cannot exclude that the alteration in this ER allele is germline and not tumor specific, since we have not sequenced the appropriate region in DNA isolated from normal tissue or peripheral blood lymphocytes from the same patient.

In conclusion, our data support the generation of abnormal ER-like mRNA from mutations occurring in the estrogen receptor gene in some human breast cancers.

## Acknowledgements

This work was supported in part by the Canadian Breast Cancer Research Initiative and the USA MRMC-DAMD17-95-1-5015. LCM is an MRC (Canada) Scientist. SAWF was supported by NIH grant CA30195. We thank Dr Pierre Chambon for his generous gifts of the GHER clones and the OR-8 cDNA.

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# APPENDIX 2

## P1-521

### XPRESSION OF EXON 6 DELETED PROGESTERONE RECEP-OR VARIANT mRNA IN NORMAL HUMAN BREAST TISSUE AND REAST TUMOURS.

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The progesterone receptor (PR) is an important prognostic marker in reast cancer as well as a marker of responsiveness to endocrine therapies. he presence of several exon-deleted PR variant mRNAs in both normal and eoplastic breast samples has recently been reported. Amongst them, a vari-nt mRNA deleted in exon 6 (D6-PR mRNA), that if translated, would en-ode a truncated PR-like protein missing the hormone binding domain and ne of the transactivating domains of the wild-type PR protein. In order to etermine whether changes in D6-PR variant expression could occur during umorigenesis, its expression was investigated by reverse transcription and CR in ten normal reduction mammoplasties samples, nine breast tumors ith high PR levels (>100 fmol/mg protein) and eight breast tumors with w PR levels (<15 fmol/mg protein), as determined by ligand binding assay. he relative expression of D6-PR to wild-type PR mRNA was lower ($P<0.01$) normal than in tumor breast samples. Moreover, a trend to lower ($P<0.1$) elative D6-PR expression was observed in high PR tumors, compared to w PR tumors. These data suggest that increased expression of D6-PR occurs uring tumor progression.

## P1-522

### UNCTIONAL CHARACTERIZATION OF NOVEL INSERTED STROGEN RECEPTORS (ER).

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Previously we identified ER-like mRNAs containing inserted sequences in me human breast tumors. Cloning these ER-like mRNAs showed: 1) RNAs containing duplication of exon 6 ER sequences. The predicted 51 Da protein is identical to wild type ER protein upto amino acid (a.a) residue 57 followed by 5 novel a.a. and truncated in the mid-E domain; 2) a mRNA ontaining duplication of exons 3+4. The predicted 85 kDa protein is entical to wild type ER except the a.a encoded by exons 3+4 are duplicated; ) a mRNA containing 69 novel nucleotides inserted between exon 5 and 6. hese sequences are inframe and encode 23 novel a.a between residues 412 nd 413 of wild type ER. The predicted protein is 69 kDa. To assess otential function of these putative ER-like proteins, appropriate expression ectors where 1) transcribed and translated *in vitro*, and their ability to bind H-estradiol determined, 2) transiently co-transfected with ERE-tk-CAT into OS-1 cells ± estradiol (10 nM) to measure transactivation potential, and 3) ansiently co-transfected with the CMV-$(ERE)_2$-CAT into COS-1 cells to ssess DNA binding potential in whole cells. Exon 6 duplicated ER did not nd estradiol nor have transactivation activity. The whole cell promoter terference assay suggested that it could bind to an ERE. The exon 3+4 uplicated ER had reduced estradiol binding and reduced ligand activated anscriptional activity. The whole cell promoter interference assay suggested at it could bind to an ERE. The 23 a.a. inserted ER had reduced estradiol nding activity and little if any transcriptional activity. The whole cell romoter interference assay suggested that it could bind to an ERE. xpression of these novel ER-like proteins in ER positive cells may modify ild type ER signal transduction. Their expression may contribute to the rogression of some breast cancers from hormone dependence to dependence and the development of endocrine resistance.

## P1-523

### CO-EXPRESSION OF VARIANT PROGESTERONE AND ESTROGEN RECEPTOR TRANSCRIPTS IN HUMAN BREAST CANCER.

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There is emerging evidence that some nuclear hormone receptors exist as a number of isoforms which may differ in their functional capacity and may interact to mediate hormone responses. This may be relevant to the phenomenon of receptor positive though hormone resistant breast cancer. The aim of this study was to compare the expression of wild-type and truncated ER and PR transcripts in a series of breast cancers in order to determine the extent of variant transcript expression in tumors known to contain different levels of the hormone dependent marker pS2. PR transcripts were measured by RT-PCR in 35 PR positive tumors and ER transcripts in 24 of these cases. The majority of tumors contained alternatively spliced PR transcripts although their abundance was extremely low and only detectable on heavily overexposed Southern blots. PR transcripts lacking exons 4 and 6 were the most abundant species noted and were present in 89% and 91% of tumors respectively. Deletions of exons 2 and exon 3 were detected in 80% and 91% of tumors respectively, and multiple exon deletions, notably deletions of exons 2+3 and 5+6 were detected in over 90% of tumors. Evidence for cryptic splicing was indicated by the detection of PR transcripts lacking half of exon 4 in 97% of tumors. Exon deleted ER transcripts were markedly more abundant than truncated PR transcripts. All of the tumors contained multiple exon deleted transcripts. Specific deletions of exons 2, 4, 5 and 7 were present in the majority of tumors. The exon 7 deleted variant was the most abundant species, expressed at levels ranging from 31 - 83% of wild type ER. There was no relationship between the expression of PR and ER truncated transcripts and expression of pS2. This study has shown that multiple alternatively spliced PR and ER transcripts are commonly co-expressed in breast cancer, although the level of expression of the PR variants is very low. The expression of truncated PR and ER transcripts was not associated with expression of pS2 and the implications of these variants for hormone responsiveness in breast cancer remains to be determined.

## P1-524

### POLYMORPHIC CAG REPEATS IN THE ANDROGEN RECEPTOR GENE IN FEMALE BREAST CANCER.

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The human androgen receptor (hAR) is an androgen modulated, DNA-binding, transcriptional-regulatory protein that is a prototypic member of the nuclear receptor superfamily and plays a role in the regulation of breast development. Epidemiological evidence suggests that women with higher lifetime "exposures" to estrogen have a higher risk of breast cancer. Estrogen promotes and maintains breast development, and androgen is antiestrogenic. The antiestrogenicity of androgen has been documented repeatedly in certain human breast cancer lines, and in advanced breast cancer, the combination of antiestrogen plus androgen therapy is superior to that of antiestrogen alone.

The transcriptional domain of the hAR is encoded by exon 1 which contains a polymorphic CAG repeat that modulates transactivation. It has been shown that longer repeats are associated with decreased transactivation, which may affect the functional estrogen:androgen balance by generating a functional hyperestrogenicity. For this reason we have chosen to assess the size of the polymorphic CAG repeat in the AR gene in breast cancer tissue. 25 fresh frozen female breast cancer samples (50 alleles) were obtained from the Manitoba Breast Tumour Bank. Genomic DNA was isolated using TRI-Reagent and amplification of the hAR was performed using nested PCR. The PCR products were separated and sized by silver staining on a 10% polyacrylamide minigel. hAR fragments of known CAG tract size (20, 22, 24, 26, 28, 32) were used as controls.

The distribution of CAG repeats in the normal population ranges from 11-33 repeats. Only 11% have tracts larger than 25 repeats and only 1% have tracts greater than 30 repeats. Our analysis showed 35% of the alleles from the breast cancer tumour samples have 26 or more repeats and 8% have more than 30 repeats. This data suggests a significant shift in the distribution of CAG repeats in the hAR gene of breast cancer tissue. The transcriptional properties of an expressed androgen receptor corresponding to a shifted CAG repeat size could lead to relative hyperestrogenicity. We are extending these studies with Class A samples which include breast cancer tissue and matching normal breast tissue. This would address the possible somatic etiology of such a shift.

# APPENDIX 3

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# EXPRESSION OF ESTROGEN RECEPTOR-*BETA* IN HUMAN BREAST TUMORS

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## ABSTRACT

The expression of a recently described novel estrogen receptor, ER–β, was detected in several human breast tumor biopsy samples and several human breast epithelial cell lines using reverse transcription and polymerase chain reaction (RT-PCR) analysis. Cloning and sequencing of the PCR product from a breast tumor confirmed the identity of the sequence with that of the ER–β mRNA previously reported in human testis. The expression of ER–β was not correlated with that of ER-α, and both ER–α positive and ER–α negative cell lines expressed ER–β mRNA. However, some breast tumors and some cell lines coexpress ER–β and ER–α mRNA. Our data support a possible role for ER–β in human breast cancer.

Estrogen signal transduction plays an important role in both normal and neoplastic mammary tissue (1). The principal mechanism by which the effects of estrogen are mediated in either normal or neoplastic target cells is via an initial interaction with the estrogen receptor (ER), a member of the steroid/thyroid/retinoid receptor gene superfamily (2). Recently, a novel ER, referred to as ER–β was cloned and characterized from human testis (3) and its rat homolog was cloned and characterized from rat prostate (4). The ER–β protein has similarities to the classical ER referred to as ER-α, in terms of structure and function. Both of these proteins have a high degree of conservation of the DNA and ligand binding domains (3), while the A/B, hinge (D) and F domains are not conserved (3,4). Transient expression assays have shown that ER–β can bind estradiol and can transactivate estrogen regulated reporter genes, although less efficiently than ER-α, and antiestrogens can inhibit this effect (3,4). Further, the tissue distribution of ER–α and ER–β although not identical appears to overlap in some cases (3,4). Therefore ER–β may be involved independently in estrogen signal transduction in some tissues but in other tissues may contribute with ER–α in estrogen signal transduction.

Estrogen has an important role in human breast cancer, however, perturbations of ER signal transduction are thought to contribute to tumor progression and the eventual development of a hormone-independent and more aggressive phenotype (5-7). The expression of ER–β in normal or neoplastic mammary tissue has not been reported, and it is important to determine if ER–β is expressed in breast cancers and therefore could potentially contribute to estrogen signal transduction in this tissue.

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## Materials and Methods

*Human tissues, cell lines and RNA extraction.*

Forty human breast tumor specimens left-over from steroid receptor assays were obtained from the Manitoba Breast Tumor Bank. Fourteen tumors were ER negative (ER < 3 fmol/mg protein), with progesterone receptor (PR) values ranging from 0 to 19.9 fmol/mg protein (median: 11.85 fmol/mg protein). Twenty six tumors presented ER levels ranging from 4.7 to 304 fmol/mg protein (median: 33.5 fmol/mg protein) and PR levels ranging from 4.1 to 764 fmol/mg protein (median: 50.5 fmol/mg protein). Total RNA was extracted using the guanidinium thiocyanate/cesium chloride method (8) as previously described (9). The human testis sample was obtained through the Manitoba Breast Tumor Bank and the MCF 10A1, MDA MB 231, T-47D and T-47D-5 cell lines were grown as previously described (10, 11). Total RNA from the cell lines and testis sample was extracted using Trizol reagent (Gibco/BRL) according to the manufacturers instructions, and the integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described (9).

*RT-PCR and Primers.*

Total RNA (1.5 μg per reaction), denatured at 65°C for 5 min, was reverse transcribed in a final volume of 15 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 μM random hexamers (Gibco/BRL) and 150 units M-MLV reverse transcriptase (Gibco/BRL). The reaction was allowed to proceed for 60 min at 37°C and was then terminated by heating at 90°C for 5 min.

One μl of this reaction was amplified by PCR in a final volume of 25 μl (if analyzed on 1.8% agarose gels stained with ethidium bromide) or 10 μl (if incorporating [α-$^{32}$P] dCTP and analyzed on 6% urea-PAGE), containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, 4 ng/μl each primer and 0.02 unit/μl of Taq DNA Polymerase (Gibco/BRL).

The primers for ER–α were: ER–α upper (sense) 5' - CAG GGG TGA AGT GGG GTC TGC TG - 3' (priming site in exon 4, nucleotides 1060 - 1083 as numbered in reference 13); ER–α lower (antisense) 5'- ATG CGG AAC CGA GAT GAT GTA GC - 3' (priming site in exon 6, nucleotides 1520 - 1543). The PCR conditions were 25 cycles of 1 min 94°C, 30 sec 60°C , and 1 min 72°C, and 20 μl of the PCR reactions were electrophoresed in agarose gels (1.8%) and visualized by ethidium bromide staining.

The primers used to amplify ER–β cDNA were: ER–β upper (sense) 5'- TGC TTT GGT TTG GGT GAT TGC - 3' (nucleotides 1164 - 1185 as numbered in reference 3); ER–β lower (antisense) 5'- TTT GCT TTT ACT GTC CTC TGC - 3' (nucleotides 1402 - 1423). The PCR conditions were 1 min 94°C, 30 sec 58°C, and 30 sec 72°C, for 30 cycles. PCR was done in the presence of [α-$^{32}$P] dCTP (3000 Ci/mmol, 1 μCi per 10 μl reaction), and 4 μl of the reaction separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and autoradiographed. In some cases, the PCR was done in the absence of radioactivity for 40 cycles, and 20 μl of the PCR reactions were electrophoresed in agarose gels (1.8%) and visualized by ethidium bromide staining.

In order to control for errors in input of cDNA used in PCR reactions, amplification of the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed in parallel using GAPDH primers (sense 5' - ACC CAC TCC TCC ACC TTT G - 3'; antisense 5' - CTC TTG TGC TCT TGC TGG G - 3') for 25 cycles of 1 min 94°C and 30 sec 52°C. PCR products were separated on agarose gels (1.8%) and visualized by ethidium bromide staining. All PCR reactions were performed at least twice in separate experiments.

PCR products from human testis and an appropriate breast tumor thought to represent ER–β were subcloned into the cloning vector, pGEM-T Easy (Promega) as previously described (12). Double stranded mini-prep DNA from two independent clones from each tissue was sequenced using a T7 Sequencing kit (Pharmacia) following the manufacturers protocol.

## Results

Forty human breast cancer biopsies were analyzed for the expression of ER–β using radioactive RT-PCR as described above. It has previously been shown that the human testis expressed ER–β mRNA at relatively high levels, and consequently RNA extracted from a sample of non-malignant human testis was used as a positive control. A 259 bp PCR product of varying intensity was detected in 70% of the breast biopsy samples analyzed (Figure 1, panel A). Several tumors displaying high, intermediate and low levels of ER–β expression using the radioactive PCR were reanalyzed using 40 cycles in a non-radioactive PCR. A 259 bp band equivalent to that found in the testis was detected in tumors displaying a strong signal in the radioactive PCR, while little if any product was detected in those tumors displaying intermediate and low signals in the radioactive PCR (Figure 1, panel B). The 259 bp signal is unlikely to result from amplification of contaminating genomic DNA, as the primers used were chosen to prime in what has been suggested to be separate exons (3). Moreover, an equivalent signal was obtained using cDNA in which the RNA had been treated with DNase I prior to reverse transcription (data not shown).

The 259 bp DNA fragments from the testis and a breast tumor sample in which a strong ER–β signal was detected were subcloned and sequenced. The tumor sequence was identical to the testis sequence, and matched that previously published for the human ER–β mRNA (3).

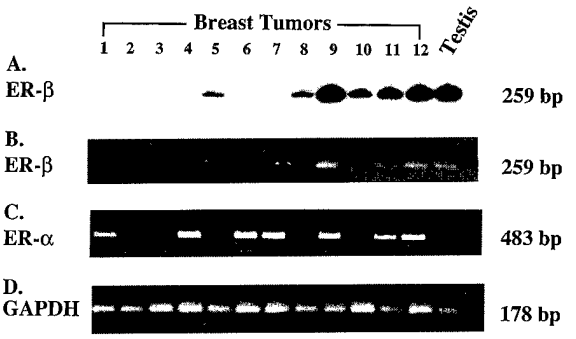


FIG. 1. **A:** Radiolabelled RT-PCR products using primers for ER–β and 30 cycles of PCR, separated on 6% PAGE containing 7M urea. Twelve human breast cancer biopsy samples and a positive control from a human testis sample are shown. The exposure was overnight with an intensifying screen. **B:** The same panel of human breast cancer biopsy samples and the human testis sample amplified with ER–β primers for 40 cycles of PCR separated on 1.8% agarose and stained with ethidium bromide. **C:** RT-PCR products using primers for ER–α from tumors and the testis sample shown in panels A and B separated on 1.8% agarose and stained with ethidium bromide. **D:** Expression of GAPDH in the tumor and testis samples shown in panels A, B and C.

Expression of ER–α mRNA in the testis and breast tumor samples was investigated using RT-PCR. The expected 483 bp DNA fragment was detected in 90% of the breast cancer biopsy samples by ethidium bromide staining (Figure 1, panel C) but no ER–α mRNA was detected in the testis sample (Figure 1, panel C). No correlation was seen between ER–α and ER–β

mRNA expression in the breast cancer biopsy samples. However, it was apparent that both genes could be expressed within the same tumor sample in some cases (see tumor samples in Figure 1, lanes 9,11,12).

To determine if the differences in level of detection were due to errors in input of cDNA used in PCR reactions, amplification of the ubiquitous GAPDH cDNA was performed in parallel using GAPDH primers (Figure 1, panel D). The results suggest similar levels of GAPDH in all samples analyzed.

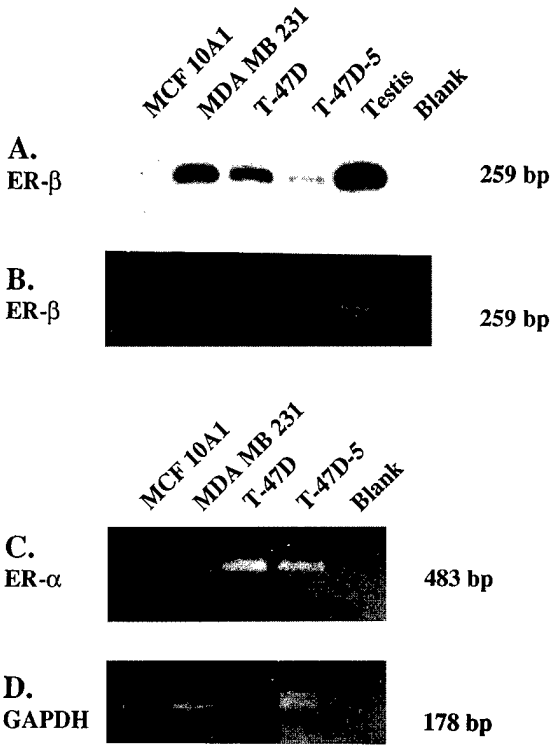


FIG. 2. **A:** Radiolabelled RT-PCR products using primers for ER–β and 30 cycles of PCR, separated on 6% PAGE containing 7M urea. Four human breast epithelial cell lines and a testis positive control are shown. The exposure was overnight with an intensifying screen **B:** The four human breast cell lines shown in panel A amplified for 40 PCR cycles using primers for ER–β separated on 1.8% agarose and stained with ethidium bromide. **C:** Expression of ER–α in the human breast cell lines shown in panels A and B using RT-PCR. **D:** Amplification of GAPDH in the four epithelial cell lines shown in panels A, B and C.

The heterogenous nature of human breast tumor samples with respect to cell type (normal and neoplastic breast epithelial cells, normal stroma, myoepithelial cells, infiltrating lymphocytes) made it difficult to determine if the ER–β mRNA was expressed exclusively in tumor cells. To address this issue we analyzed the expression of ER–β mRNA in several human breast epithelial cell lines, including breast cancer cell lines. The expected 259 bp band was detected at varying levels in all breast epithelial cell lines following autoradiography of radiolabelled RT-PCR products (Figure 2, panel A), and was detected by ethidium bromide staining of nonradioactive RT-PCR products obtained from T-47D and MDA MB 231 cells (Figure 2, panel B).

ER–β mRNA was detected in cell lines which were both ER–α positive (T-47D, T-47D-5) and ER–α negative (MDA MB 231, MCF 10A1). ER–α mRNA expression was determined by RT-PCR (Figure 2, panel C). Differences in signal are unlikely to be due to differences in input cDNA as shown by the equivalent GAPDH signal observed in all samples (Figure 2, panel D).

## Discussion

The data presented in this paper provide evidence for the expression of the ER–β gene in human breast epithelial cells. Our results are the first, to our knowledge, to address the issue of ER–β expression in either normal or neoplastic breast tissue or cells. ER–β mRNA was detected in both human breast tumor biopsy samples and human breast epithelial cell lines growing in culture. The level of expression of this gene appeared to vary amongst tumor samples and between cell lines, but the expression was not correlated with the expression of ER–α mRNA. Indeed both ER–α positive (T-47D) and ER–α negative (MDA MB 231) cell lines, as determined by ligand binding assays (14) and RT-PCR analysis (Figure 2, panel C), were found to express relatively high levels of ER–β mRNA. Interestingly, the non-tumorigenic, apparently 'normal' human mammary epithelial cell line, MCF 10A1 (11), contained detectable ER–β mRNA suggesting the possibility that ER–β may be expressed in normal human mammary epithelial cells. The ER–β has been shown to have some functional similarities to the ER–α in that it can bind estradiol-17β and activate an ERE- regulated reporter gene construct, and antiestrogens can inhibit ER–β activity in these assays (3,4). However, reduced potency of estrogen activation of ER–β with respect to ER–α was noted (3), and since marked differences between these two ERs in the A/B , hinge (D) and F domains exist the assay systems previously used may not be optimal for ER–β in terms of the cell type, the promoter and possibly the ligand (3). The detection of relatively high levels of ER–β mRNA in MDA MB 231 breast cancer cells, which have previously been shown to be ER–α negative by ligand binding assays and in this paper by RT-PCR analyses, questions the

functional significance of ER–β expression in these cells at least with regard to mediating an 17β-estradiol signal. However, the lack of any clearly defined function for this protein as well as possible different ligand preferences (15) severely limits the interpretation of such data.

Although we found no correlation between ER–α and ER–β expression, some tumors and some cell lines were found to co-express these two genes. These data are consistent with previous findings (3,4) in which ER–β expression was found to have an over-lapping but non-identical tissue distribution to ER-α. While the radioactive PCR used to screen the breast tumor biopsy samples is a highly sensitive method likely to detect very low levels of expression of ER–β, several tumors presented a strong signal equivalent to that seen in the testis sample using both radioactive PCR and by ethidium bromide staining of PCR products. Our data suggest that ER–β may have a role in breast cancer cells, and this role may be expected to differ depending on the presence or absence of expression of the classical ER-α. Further, given the similarities and differences so far identified between these two gene products our results suggest that an involvement of ER–β in estrogen signal transduction or altered estrogen signal transduction in breast tissue will have to be considered.

## Acknowledgments

This work was supported by grants from the U.S. Army Medical Research and Materiel Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). L. C. M. is an MRC Scientist, P. H. W. is a Medical Research Council of Canada (MRC) Clinician-Scientist, E. L. is a recipient of a USAMRMC Postdoctoral Fellowship Award. The authors thank Caroline Cumins-Leygue and Helen Bergen for laboratory assistance with the cell culture.

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# APPENDIX 4

# Estrogen receptor-β mRNA variants in human and murine tissues

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## ABSTRACT

ER-β mRNA splice variants have been identified in human breast tumors as well as normal human and mouse ovarian, uterine and mammary tissues. In both species transcripts deleted in exons 5 or 6, or 5+6 have been characterized by RT-PCR followed by cloning and sequencing. In mouse tissues an ER-β transcript containing 54 nucleotides inserted in frame between exons 5 and 6 was identified. Interestingly, no equivalent of the mouse inserted transcript was detected in any of the four human tissues analyzed.

Recently, the cDNA of a second estrogen receptor, ER-β, was cloned and sequenced (1) from the rat, the human (2) and the mouse (3). Northern analysis of RNA isolated from mouse ovary demonstrated the presence of multiple mRNA species for ER-β (3) suggesting the possibility that variant ER-β proteins might exist. To investigate whether ER-β variant mRNAs might be expressed in human as well as murine tissues, a reverse-transcription polymerase chain reaction (RT-PCR) analysis was undertaken which demonstrated the presence of variant ER-β mRNAs in both species.

## Materials and Methods

*Tissues and RNA extraction.*

Human breast tumor specimens left-over from steroid receptor assays were obtained from the Manitoba Breast Tumor Bank, and three non-malignant human uterine hysterectomy samples were obtained from the Department of Obstetrics and Gynecology, Health Sciences Centre, Winnipeg, Canada. Total RNA was extracted using the guanidinium thiocyanate/cesium chloride method as previously described (4). Four non-malignant human ovarian samples from two pre-menopausal and two post-menopausal women were obtained through the Ovarian Tissue Bank at the Institut du Cancer de Montreal, Centre de Recherche Louis-Charles Simard, Montreal, Canada. Four normal human breast tissues from reduction mammoplasties of pre-menopausal women were obtained through the Manitoba Breast Tumor Bank. Total RNA from the ovarian and normal breast tissue samples was extracted using Trizol reagent (Gibco/BRL) according to the manufacturer's instructions.

Mouse uteri and ovaries were obtained from four female mice aged 8 to 9 weeks, and mammary tissues were obtained from two adult lactating female mice. Total RNA was extracted using Trizol reagent (Gibco/BRL) according to the manufacturer's instructions.

Integrity of RNA was confirmed by denaturing gel electrophoresis as previously described (5).

*RT-PCR and Primers.*

Total RNA (1.5 μg per reaction) was reverse transcribed as previously described (6). One μl of this reaction was amplified by PCR incorporating $^{32}P$ in a final volume of 10 μl, and 4 μl of this reaction separated on 6% denaturing polyacrylamide gels and autoradiographed as previously described (6).

All ER-β exons are defined in this report by analogy to the human ER-α exon structure (7):

Human primer set one: hER-β exons 4 and 7: hER-β-4 (sense) 5'-GGC CGA CAA GGA GTT GGT A-3' (priming site in exon 4, nucleotides 762-780 as numbered in reference (2)); hER-β-7 (antisense) 5'-TCC ATG CCC TTG TTA CTC G-3' (priming site in exon 7, nucleotides 1262-1280). The PCR conditions were 30 cycles of 1 min 94°C, 30 sec 60°C , and 1 min 72°C.

Human primer set two: hER-β exons 5 and 6: hER-β-5 (sense) 5'-GCT GTT GGA TGG AGG TGT TA-3' (priming site in exon 5, nucleotides 857- 876); hER-β-6 (antisense) 5'-CTT GAA GTA GTT GCC AGG AG-3' (priming site in exon 6, nucleotides 997-1016). The PCR conditions were 30 cycles of 30 sec 94°C, 30 sec 60°C, and 30 sec 72°C.

Mouse primer set one: mER-β exons 4 and 8: mER-β–4 (sense) 5'-CTG AAC AAA GCC AAG AGA-3' (priming site in exon 4, nucleotides 600-617 as numbered in reference (3)); mER-β-8 (antisense) 5'-GCT CTT ACT GTC CTC TGT CG-3' (priming site in exon 8, nucleotides 1417-1436). The PCR conditions were 35 cycles of 1 min 94°C, 1 min 60°C , and 2 min 72°C.

Mouse primer set two: mER-β exons 5 and 6: mER-β-5 (sense) 5'-GCT GAT GGT GGG GCT GAT GT-3' (priming site in exon 5, nucleotides 890-909); mER-β-6 (antisense) 5'-ATG CCA AAG ATT TCC AGA AT-3' (priming site in exon 6, nucleotides 993-1012). The PCR conditions were 35 cycles of 1 min 94°C, and 30 sec 60°C.

PCR products from human breast tumors and mouse mammary tissues were subcloned into the cloning vector, pGEM-T Easy (Promega) following the manufacturer's instructions. Double stranded DNA from at least two independent clones from each tissue was sequenced using a T7 Sequencing kit (Pharmacia) following the manufacturer's protocol. All RT-PCRs were carried out at least twice for each sample analyzed.

## Results and Discussion

Previously the presence of ER-β mRNA was identified in some human breast tumor samples (6). The RT-PCR analysis employed a primer set which by analogy to the ER-α exonic structure would anneal to sequences corresponding to exons 7 and 8 of the human ER-β cDNA. Numerous splicing variants of the human ER-α mRNA have been identified to date (see (8) for review), and it was of interest to determine if similar splice variants could be detected in the ligand binding domain of the ER-β mRNA in human breast tumors. Using a primer set which by analogy to human ER-α would anneal to sequences located in exons 4 and 7 of the human ER-β cDNA, RT-PCR analyses were undertaken using RNA isolated from four separate human breast tumor samples which had previously been shown to express ER-β mRNA by RT-PCR using an exon7/8 primer set (6). The results presented in Fig. 1, show the presence of the expected 519 bp wild-type ER-β product as well as several smaller sized PCR products.

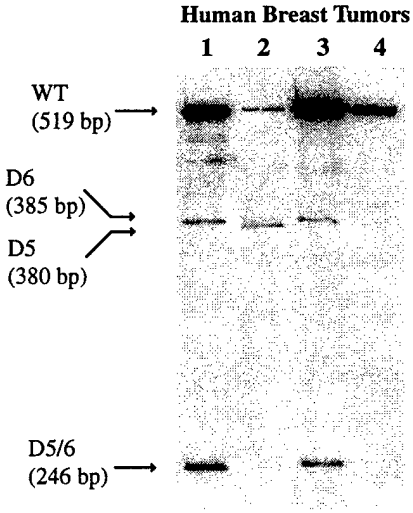


FIG. 1. Detection of wild-type ER–β and ER–β variant mRNAs in human breast tumor tissues. Total RNA extracted from 4 different human breast tumors (1-4) was reverse transcribed and PCR amplification was performed using primers located in exons 4 and 7. PCR products migrating at the sizes of 519 bp, 385 bp, 380 bp and 246 bp were subsequently cloned, sequenced and identified as corresponding to ER–β wild-type (WT), exon 6-deleted variant (D6), exon 5-deleted variant (D5) and exon 5-6-deleted variant (D5/6) cDNAs, respectively.

Cloning and sequencing of the smaller sized products (Fig. 2) revealed deletions of nucleotides 812-950, 951-1084 and 812-1084 (numbered as in reference (2)) which by analogy to ER-α would be a precise exon deletion of exon 5, exon 6, and exon 5 plus 6, respectively. To determine if such deletions occurred only in human breast tumor tissue, RNA extracted from several normal breast, uterine and ovarian tissue samples was analyzed (Fig. 3, top panel). The same tissues from the mouse were analyzed in parallel, using a mouse ER-β primer set located in putative exons 4 and 8 (Fig. 3, bottom panel; all exons, mouse and human, are numbered according to the human ER-α structure (7)). All human tissues analyzed expressed ER-β variant mRNAs similar to those identified in breast tumors. The expected wild-type product of 837 bp was detected in all mouse tissues, as were several smaller sized PCR products. Cloning and sequencing of the 698 bp fragment identified deletions of nucleotides 829-967, which by analogy to the mouse ER-α would be a precise deletion of exon 5; and deletions of nucleotides 829-1101 representing deletion of exons 5 and 6 (Fig. 2, nucleotides numbered as in reference (3)). The 703 bp band was found to correspond to an exon 6 deleted ER-β transcript.

These data support the expression of exon deleted splice variants for ER-β similar to those for ER-α, in both human and murine tissue samples. The exon deleted splice variants identified in this study are out-of-frame and would be expected to encode C-terminally truncated ER-β proteins, which are unlikely to bind ligand.

In contrast to what was observed in human tissues, all murine tissues analyzed presented a prominent ER-β 891 bp PCR product which was larger than the expected wild type ER-β product of 837 bp (Fig. 3, bottom panel). Sequencing of the larger PCR product revealed an insertion of 54 nucleotides between nucleotides 967 and 968 (as numbered in reference 3), which by analogy to ER-α would be precisely inserted between the splice junction of exons 5 and 6. Identical results were obtained when

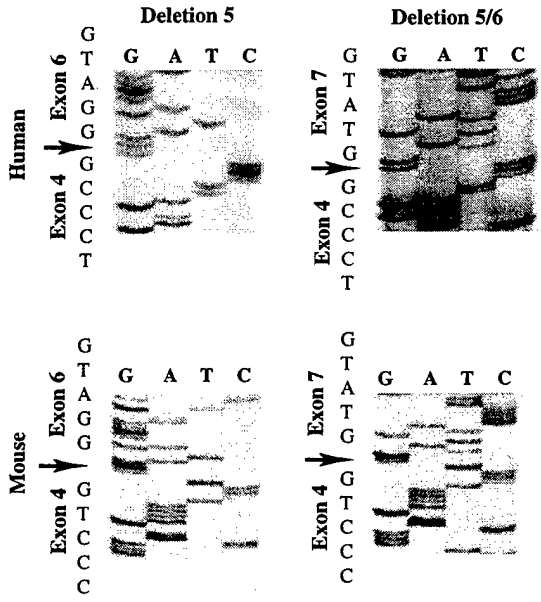


FIG. 2. Sequencing of exon 5-deleted and exon 5/6-deleted ER–β variants in human and murine tissues. Top panels: Sequencing of PCR products obtained by amplification of human breast tumor cDNAs using primers in exons 4 and 7 and migrating at the sizes of 380 bp and 246 bp (see Fig. 1) showed a perfect junction between exon 4 and 6 (deletion 5), and exon 4 and 7 (deletion 5/6), respectively. Bottom panels: Sequencing of PCR products obtained by amplification of murine breast tissue cDNAs using primers in exons 4 and 8 and migrating at the sizes of 698 bp and 564 bp (see Fig. 3) showed a perfect junction between exon 4 and 6 (deletion 5), and exon 4 and 7 (deletion 5/6), respectively.

the starting RNA samples were enriched for polyadenylated transcripts using oligo-dT attached to magnetic beads (data not shown), suggesting that the inserted transcript represented an authentic mRNA species. The sequence of this insertion is shown in Figure 4. This insertion is inframe and the predicted amino acids are shown in Figure 4 also. While this work was in progress the presence of a 54 bp inserted ER-β transcript in rat tissues was published (9). The sequence of the 54 nucleotide insertion in the murine ER-β transcript is identical to that published for the rat except for a T→C change at nucleotide position 36 ( 1 = start of the 54 nucleotide insert), which would result in a Met→Thr substitution in the mouse protein.

Because the initial screening of human tissues using the exon 4/7 primer set failed to reveal an analogous human ER-β transcript containing an insertion between exons 5 and 6, reanalysis of human and mouse tissues was undertaken using primer sets located in exons 5 and 6 of either the human or the mouse ER-β. While the inserted ER-β transcript was easily detected as a 177 bp PCR product in all murine tissues analyzed, only the expected 160 bp PCR product corresponding to the human wild-type ER-β mRNA was detected in the normal human tissues (Fig. 5), nor was an inserted ER-β variant detected in 10 human ER-β RNA positive breast tumor samples (6) (data not shown). The data shown in Fig. 5 suggest that the inserted transcript is predominant in both mouse mammary gland and uterus, while similar levels of each transcript occur in the mouse ovary. Measurement of relative expression using such an approach has been validated previously (10, 11).

A possible mechanism associated with the frequent inclusion of the inserted sequences in mouse ER-β transcripts may be the presence of several putative exonic splicing enhancer sequences within the insertion sequences (12, 13). Both purine rich motifs and A/C-rich splicing enhancer sequences are present (brackets in Fig. 4). The frequent inclusion of the inserted sequences in mouse ER-β transcripts, and its presence in several tissues at comparable levels to the wild-type transcript suggest that the protein encoded by the inserted transcript has a functional role, at least, in the mouse and rat (9). The putative function of a protein encoded by the inserted mouse ER-β transcript is unknown, however, since the insertion is in the middle of the ligand binding domain, it may either disrupt binding completely or result in a different ligand binding specificity and/or affinity. Further, the insertion may effect the three dimensional structure

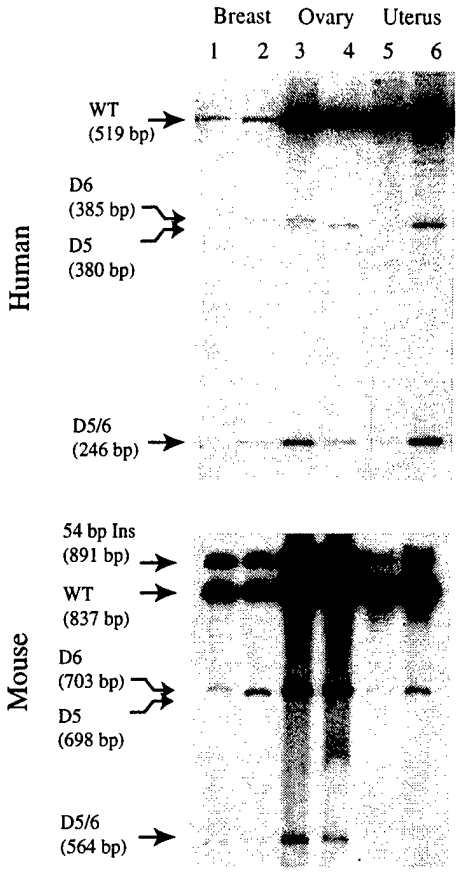


FIG. 3. Detection of wild-type ER–β and ER–β variant mRNAs in normal human and murine tissues. Total RNA extracted from normal human (top panel) or mouse (bottom panel) breast tissues (1-2), ovaries (3-4) and uteri (5-6) was reverse transcribed and PCR amplification was performed using primers located in exons 4 and 7 (human) or in exons 4 and 8 (mouse). PCR products obtained in human tissues migrated at the sizes of 519 bp, 385 bp, 380 bp and 246 bp corresponding to ER–β wild-type (WT), exon 6-deleted (D6), exon 5-deleted (D5) and exon 5-6-deleted (D5/6) cDNAs, respectively. PCR products obtained in mouse tissues and migrating at the sizes of 891 bp, 837 bp, 703 bp, 698 bp, and 564 bp were identified as corresponding to a 54 bp inserted ER–β variant (54 bp Ins), ER–β wild-type (WT), exon 6-deleted variant (D6), exon 5-deleted variant (D5) and exon 5-6-deleted variant (D5/6), respectively.

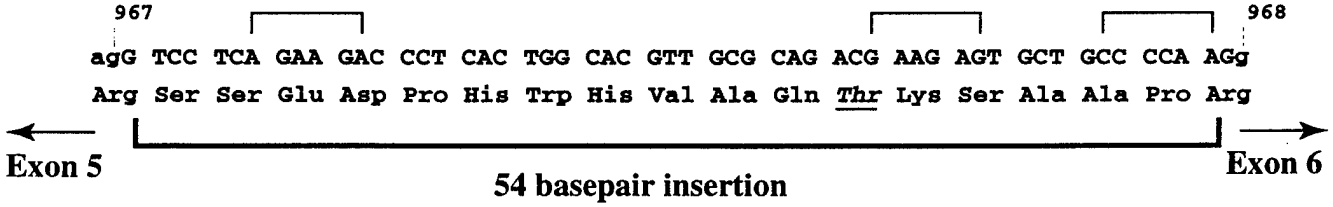


FIG. 4. 54 bp insertion: nucleotide and amino-acid sequences. The sequence of the inserted 54 bp is indicated in upper case letters. Nucleotides corresponding to the published mouse ER–β cDNA sequence, indicated in lower case letters are numbered according to reference 3. Brackets indicate putative exonic splicing enhancer sequences (12,13). Predicted amino acid composition of the insert is shown. The underlined amino acid corresponds to the substitution observed between mouse inserted sequence and the recently described rat inserted sequence (9).

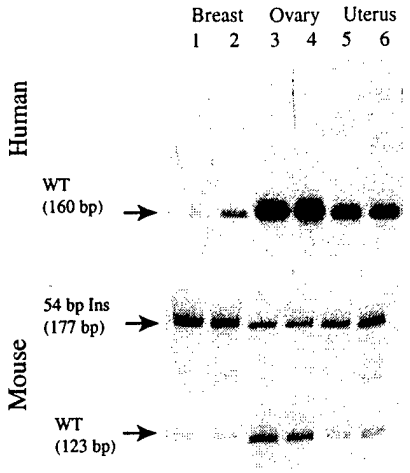


FIG. 5. Amplification of human and murine cDNAs using exon 5 and 6 primers. Total RNA extracted from normal human (top panel) or mouse (bottom panel) breast tissues (1-2), ovaries (3-4) and uteri (5-6) was reverse transcribed and PCR amplification was performed using primers located in exons 5 and 6. The PCR product obtained in human tissues migrated at the size of 160 bp corresponding to ER–β wild-type (WT). PCR products obtained in mouse tissues migrated at the sizes of 177 bp and 123 bp, corresponding to a 54 bp inserted ER–β variant (54 bp Ins) and ER–β wild-type (WT), respectively.

of the E-domain such that alterations in dimerization, transctivation and interaction with co-regulators may also occur. This could result in the inserted ER-β having a regulatory function on the wild-type ER-β as previously suggested (9), or may completely alter its ability to heterodimerize and effect the activity of ER-α (14). The lack of detection of a similar inserted ER-β transcript in human tissues may be due to hormonal differences at the time of tissue collection between the mouse and human subjects, or to a real species difference in alternative splicing. In the latter case, differences in alternative splicing between mouse and human with regard to a steroid hormone receptor have been previously documented (15, 16). Similarly inserted sequences within the ligand binding domain of ER-α have also been reported (17). In contrast to the inserted ER-β mRNA, the inserted ER-α mRNA was detected in one human breast tumor sample, and was due to a point mutation in one allele of the human ER-α gene present in the breast tumor (18).

In summary, splice variants of ER-β mRNA have been characterized in several mouse and human tissues. Although splice variants of ER-β occur in both normal and neoplastic tissues, a species specific difference in the expression of an inserted splice variant was detected.

## Acknowledgements

This work was supported by the U.S. Army Medical Research and Materiel Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). LCM is a Medical Research Council of Canada (MRC) Scientist, PHW is a MRC Clinician-Scientist, EL is a recipient of a USAMRMC Postdoctoral Fellowship. The authors thank Heidi Hare for excellent technical assistance.

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# APPENDIX 5

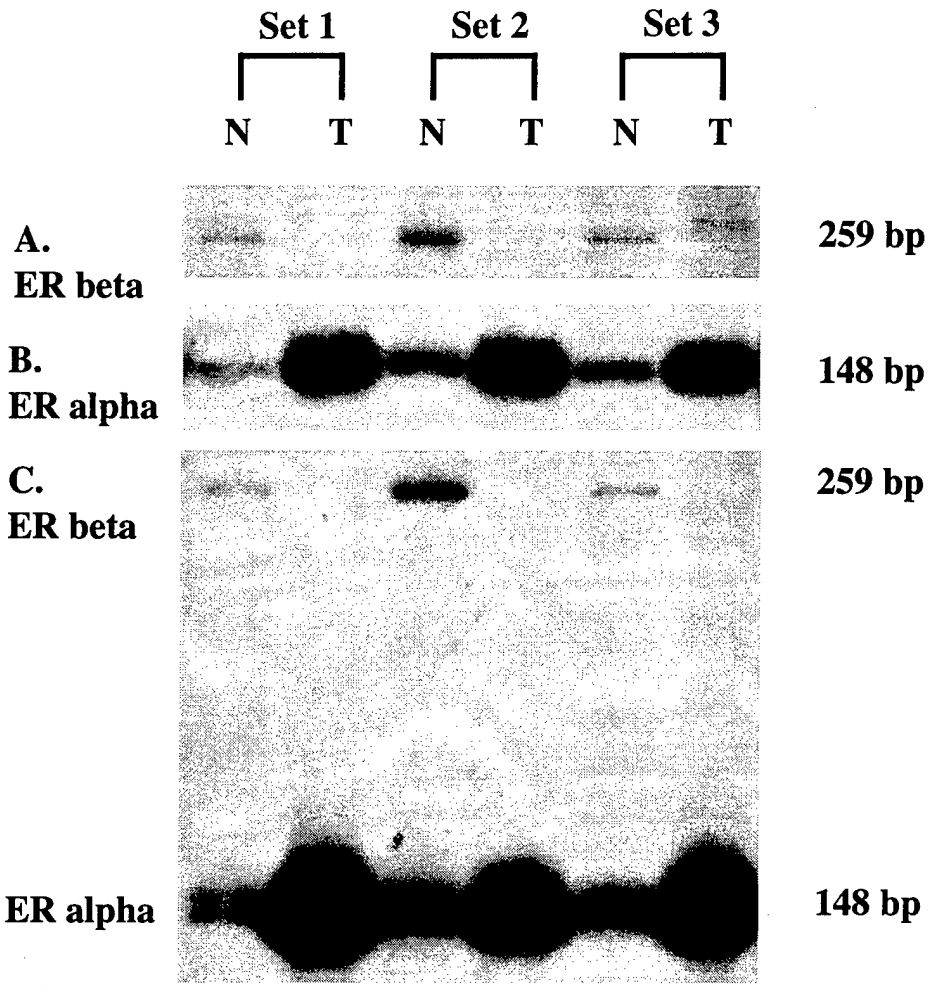



**FIG. 1: Matched human breast normal/tumor samples analyzed for ER-alpha/ER-beta mRNA expression by multiplex PCR.** RNA obtained from three matched human breast normal and tumor samples were analyzed for the relative expression of ER-alpha and ER-beta. **A.** The three matched samples analyzed using PCR primers for ER-beta. **B.** The same three matched samples analyzed using PCR primers for ER-alpha. **C.** The same three matched normal/tumor samples analyzed using PCR primers for both ER-alpha and ER-beta in a multiplex PCR.

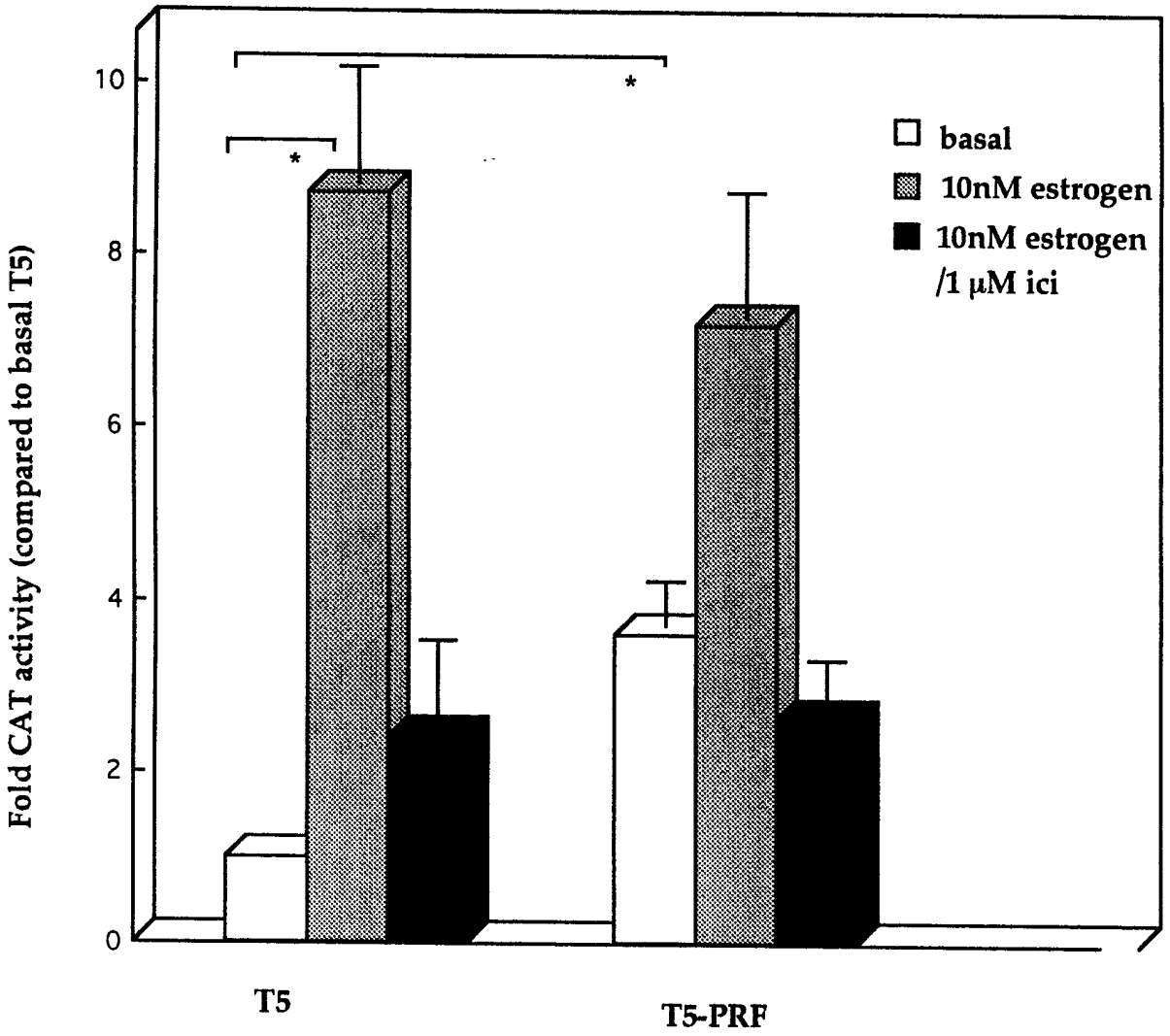



**Figure 2 ER transcriptional activity.** T5 and T5-PRF cells were transfected and CAT assays performed as described in "Methods". Results are expressed as fold CAT activity compared to T5 basal (arbitrarily set at 1.0). *$p<0.05$, students' t-test compared to T5 basal. Results represent mean $\pm$SEM, n=7.

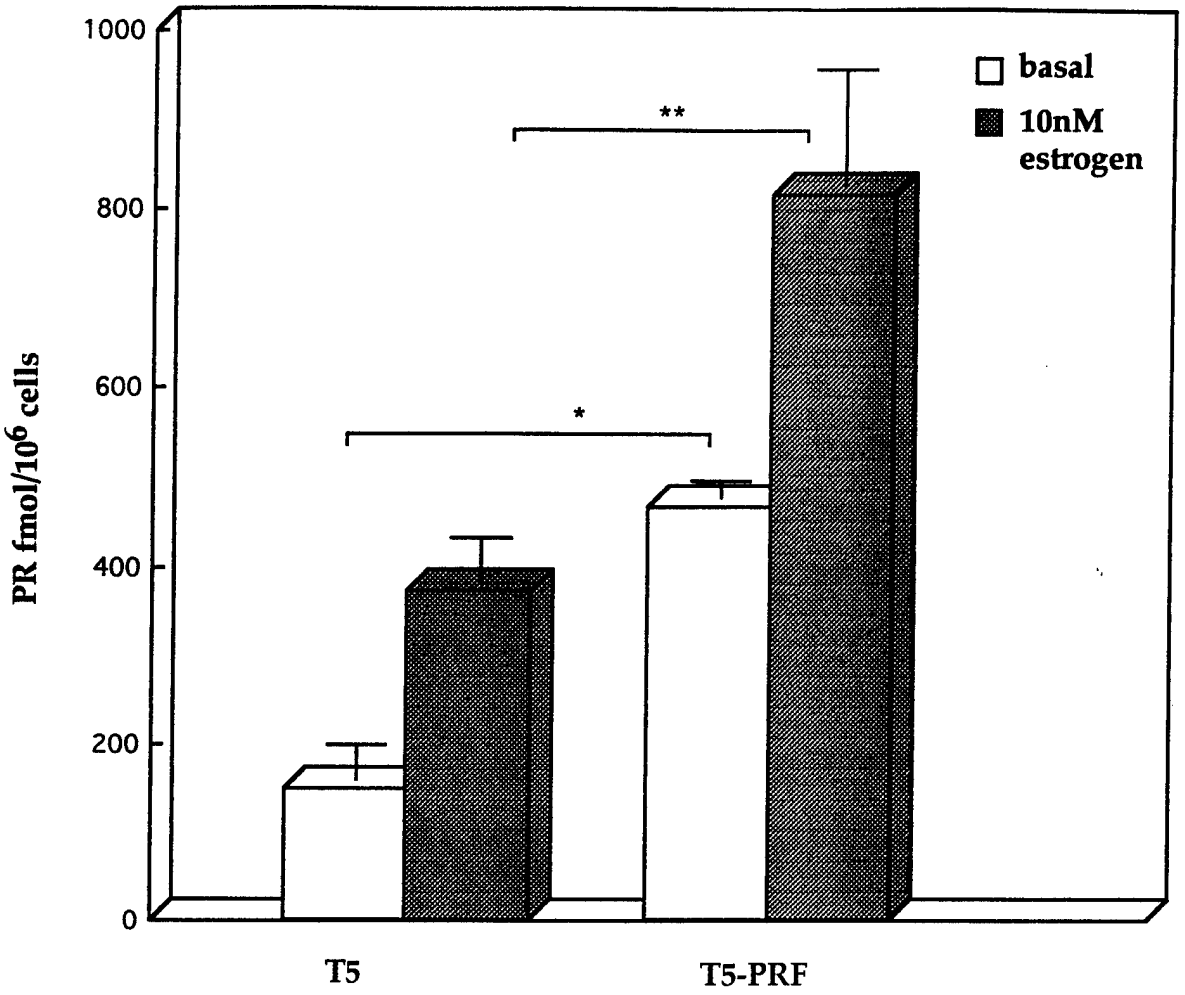



**Figure 3 Progesterone receptor levels.** PR levels were determined by whole cell binding as described in "Methods". PR levels are expressed as fmol PR/106 cells and results represent mean +/- SEM, n=3. * $p < .001$, **$p<0.05$ student's t-test.

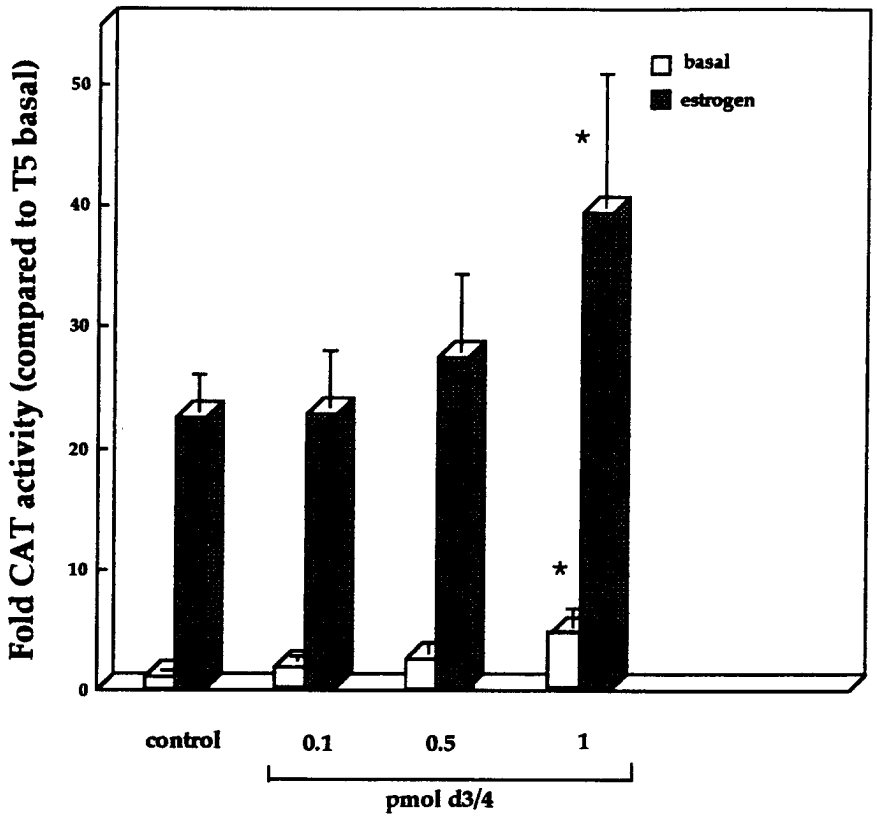



**Figure 4 Transient transfection of d3/4 expresson vector into T5 cells.**

Cells were grown in PRF-DMEM as described in Methods and transfected with 5μg ERE-tk-CAT expression vector, 5μg pCH110 (β-gal expression vector) along with the appropriate amount of d3/4 ER expression vector. Cells were treated with vehicle or 10nM estradiol for 24 h, harvested and CAT assays performed as described in Methods. Results represent mean ± sem, n=2-5. *, $P < 0.05$, Wilcoxon's Rank Sum Test (compared to T5 basal).

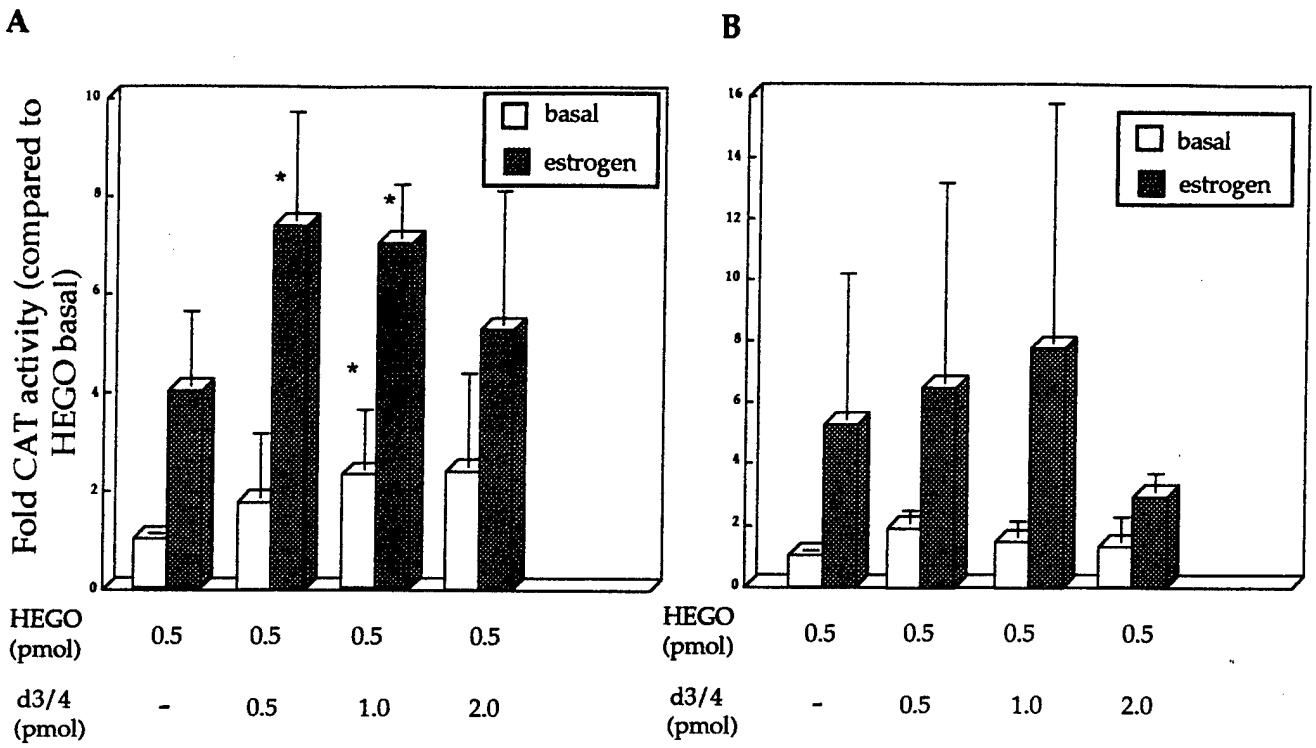
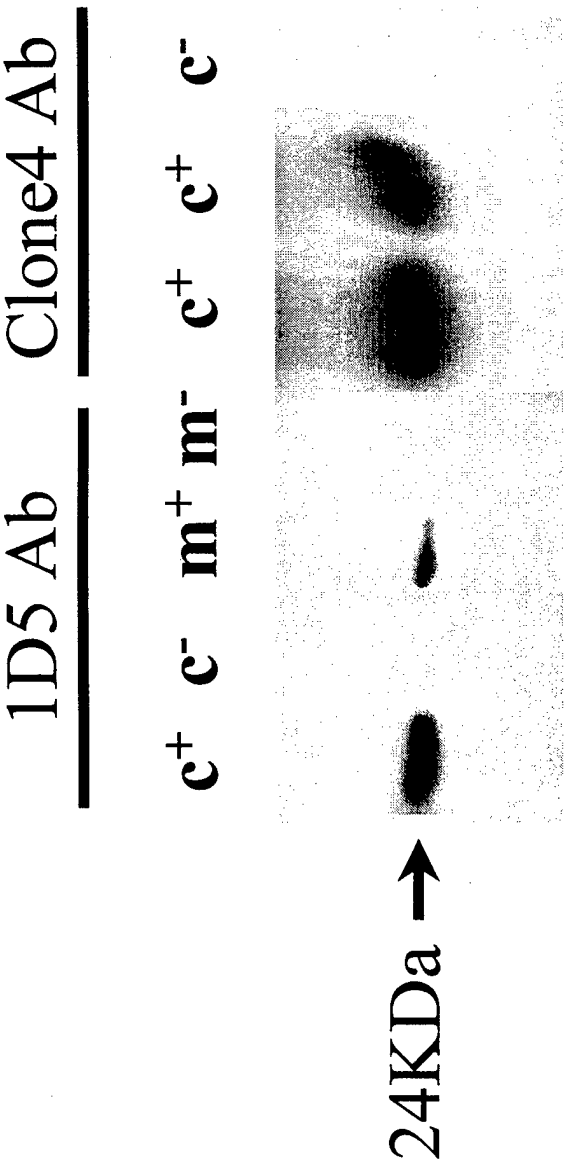



**Figure 5 Activity of d3/4 in ER negative cells. A.** MDA-MB-231 cells were transfected with 5 μg EREtkCAT, 1 μg pCH110, 0.5 pmol HEGO +/- 0.5-2 pmol d3/4 +/- vector to a total of 30μg DNA/dish. Cells were treated with 10nM estrogen for 24 h or vehicle alone as control. Results are expressed as fold CAT activity compared to basal HEGO activity arbitrarily set as 1.0. Histograms represent mean +/- sem, n=5-7. *$p<0.05$ Wilcoxon's Rank Sum. **B.** MCF10A1 cells were transfected similarily. Histograms represent mean ± sem, n=3.

Legend to Figure 6:

Whole cell lysates from Cos-1 cells transfected with a clone 4 expression vector (c+) or vector alone (c-), and from MCF 7 cells transfected with a clone 4 expression vector (m+) or vector alone (m-), were analyzed by Western blotting. A similar approximately 24 kDa protein (arrow) was detected in clone 4 transfected cells only and not vector alone transfected cells, when either an N-terminal specific ER-$\alpha$ antibody (1D5) or an antibody raised to a synthetic peptide to the novel clone 4 amino acid sequences (clone 4 Ab) was used.

Fig 6



# APPENDIX 6

# COMPARISON OF ESTROGEN RECEPTOR-α VARIANT mRNA EXPRESSION IN PRIMARY HUMAN BREAST TUMORS AND THEIR MATCHED LYMPH NODE METASTASES.

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**ABSTRACT.** Previously we had shown that the relative expression of a clone 4 truncated estrogen receptor-α variant mRNA was significantly increased in primary breast tumors which had concurrent lymph node metastases compared to primary breast without lymph node metastases. In this study we have measured and compared the relative expression of a clone 4 truncated, an exon 5 deleted and an exon 7 deleted estrogen receptor-α variant mRNA in 15 primary breast tumor samples and their matched concurrent lymph node metastases. There were no differences between the primary and the matched secondary tumors in the relative expression of these three variants mRNA. Furthermore, the pattern of all deleted estrogen receptor-α variant mRNA appeared conserved between the primary and the matched secondary tumors.

**INTRODUCTION.**

Multiple estrogen receptor-α (ER) mRNA species have been identified in human breast cancer samples (1, 2). The significance of these variant transcripts remains unclear. While the ability to detect variant ER proteins encoded by such variant transcripts remains controversial (3-5), alteration of expression of some variant ER mRNAs was found to occur during both breast tumorigenesis (6, 7) and breast cancer progression. With regard to the latter, we have shown previously that the expression of the truncated, clone 4 variant ER mRNA (8) relative to wild type ER mRNA was significantly increased in a group of primary breast tumors with poor prognostic features compared to a group of primary breast tumors with good prognostic features (9). "Poor" prognostic features included the presence of lymph node metastases at the time of surgery, large tumor size, lack of progesterone receptor (PR) expression and high proliferative index, while "good" prognostic features were lack of nodal involvement, small tumor size, PR positivity and low proliferative index. This suggested that altered ER variant expression may be a marker of a more

aggressive phenotype. We have investigated this possibility further by comparing the over-all pattern of deleted ER variant expression between matched primary tumors and their concomitant lymph node metastases, as well we have compared the relative level of expression of several individual ER variant mRNAs in these matched tumor samples.

## MATERIALS AND METHODS.

### Tumor Selection and RNA Isolation.

Sections from 15 frozen primary human breast tumor samples and their matched frozen lymph node metastases were provided by the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). For the primary tumor samples, the ER levels determined by ligand binding assays ranged from 0.8 fmol/mg protein to 89 fmol/mg protein with a median value of 17.5 fmol/mg protein. Thirteen tumors were ER+ and 2 were ER- ( > 3 fmol/mg protein is ER+ ). PR levels determined by ligand binding assays ranged from 2.9 fmol/mg protein to 112 fmol/mg protein with a median value of 12.6 fmol/mg protein. Nine tumors were PR+ and 6 were PR- (> 10 fmol/mg protein is PR+). ER and PR values were only available for 4 of the lymph node metastases and the ER and PR status as defined by ligand binding was not different to their matched primary tumor. RNA was extracted from the sections using Trizol reagent (Gibco/BRL) according to the manufacturer's instructions.

For validation of comparison of triple primer ploymerase chain reaction (TP-PCR) and RNase protection assays human breast tumor specimens (25 cases) were also obtained from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Twenty tumors were ER positive, as determined by ligand binding assay, with values ranging from 4.5 to 311 fmol/mg protein (median= 93 fmol/mg). The five remaining cases were ER negative, as determined by ligand binding assay, with values ranging from 0 to 1.8 fmol/mg protein

(median 0.9 fmol/mg). Total RNA was extracted from frozen tissues using guanidium-thiocyanate as previously described (10). the integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described (10).

**RNase Protection Assay.**

Antisense riboprobes spanning the point at which the clone 4 truncated ER mRNA sequence diverges from the wild type ER mRNA sequence (8) were synthesized as previously described (11). The level of clone 4 truncated ER mRNA and WT-ER mRNA in 10 µg total RNA was determined using an RNase Protection Assay kit (RPA II, Ambion) following the manufacturer's instructions. Briefly, RNA was denatured at 80°C for 5 min in the presence of 5 x $10^5$ dpm of $^{32}$P-labelled riboprobe, then hybridized at 42°C for 16 hours. Following RNase digestion, samples were electrophoresed on 6% acrylamide gels containing 7 M urea, dried and autoradiographed.

To quantify C4 and WT-ER mRNAs within breast tumor samples, a standard curve was established in each assay. Clone 4 and WT-ER mRNAs (30, 125, 500 pg clone 4 RNA and 125, 500, 2000 pg WT-ER RNA) synthesized using T7 RNA Polymerase were purified on a Sephadex G-50 column and quantitated spectrophotometrically. WT-ER RNA was transcribed from linearized pHEO, which contains the entire WT-ER coding sequence but is missing the 3'-untranslated portion of the ER mRNA (kindly provided by P. Chambon, (12)). Full-length C4 RNA was transcribed from linearized pSK-C4 (8). Standard RNAs were analyzed together in the same assay as the breast tumor mRNAs. Bands corresponding to the clone 4 variant ER mRNA and WT-ER mRNA protected fragments were excised from the gel and counted after addition of 5 ml scintillant (ICN Pharmaceuticals, Inc., Irvine, CA) in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA). For each

sample, absolute amounts of clone 4 and WT-ER mRNA were determined from the standard curve.

**Reverse transcription, PCR and Triple Primer (TP-PCR).**

For each sample, one microgram of total RNA was reverse transcribed in a final volume of 15 µl as described previously (7). One microliter of the reaction mixture was taken for subsequent amplification.

The primers and PCR conditions for the long range PCR were as previously described (13). The primers and PCR conditions for measuring the relative expression of exon 5 deleted and exon 7 deleted ER transcripts relative to the wild type ER transcripts were as previously described (7).

The TP-PCR conditions were similar to those previously described (6) with some modifications. ERU (5'-TGTGCAATGACTATGCTTCA-3', sense, located in WT-ER exon 2; 792-811, as numbered in (12)) and ERL (5'-GCTCTTCCTCCTGTTTTTAT-3', antisense, located in wild type ER exon 3; 921-940) primers allowed amplification of a 148 bp fragment corresponding to wild type ER mRNA. Clone 4 specific primer (C4L, 5'-TTTCAGTCTTCAGATACCCCAG-3', antisense; 1315-1336, as numbered in reference (8)) was chosen as spanning the only region of the C4 unique sequence that does not present any homology with repetitive LINE-1 sequences (8). ERU and C4L allowed amplification of a 536 bp fragment corresponding specifically to clone 4 truncated ER variant mRNA.

PCR amplifications were performed in a final volume of 10 µl, in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl2, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/µl of each primer (ERU, ERL and C4L), 1 unit of Taq DNA polymerase (GIBCO-BRL) and 10 nM of dCTP [$\alpha$-$^{32}$P] (ICN Pharmaceuticals Inc, Irvine, California). Each PCR consisted of 30 cycles (1 minute at 94°C, 30 seconds at 60°C and 1 minute at 72°C) using a

Thermocycler (Perkin Elmer). 4 µl of the reaction was then denaturated by addition of 6 µl of 80% formamide buffer and boiling before electrophoresis on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C with two intensifying screens for 2 hours.

**Quantification of RT-PCR and TP-PCR.**

Bands corresponding to the variant ER mRNA and WT-ER mRNA were excised from the gel and counted after addition of 5 ml scintillantin a scintillation counter. The variant signal was expressed as a percentage of the wild type ER signal. It should be noted that the percentage obtained reflects the relative ratio of the variant to wild type ER PCR product and does not provide absolute initial mRNA levels. Validation of this approach has been described previously (6, 7, 14, 15). For each sample in the comparison of RNase Protection Assay with TP-PCR assays, at least two independent PCR assays were performed. For comparisons of matched primary and secondary breast tumor samples, at least two and in most cases three independent PCR reactions were performed and the mean determined.

The statistical significance of differences in the relative levels of expression of any single variant between primary tumor and lymph node metastasis was determined using the Wilcoxon signed rank test.

**RESULTS.**

A frequently expressed clone 4 truncated ER variant was previously found to be significantly elevated in primary breast tumors with concurrent lymph node metastases compared to those without (9). In this study we asked the question if ER variant expression was altered between primary breast tumors and their matched, concurrent lymph node metastasis. In our previous study we used RNase protection analyses to measure wild type and variant ER

mRNA expression. However, for practical reasons of small amounts of tissue sample available in this current study we have used a previously described TP-PCR assay (6) to measure the relative expression of the clone 4 truncated ER mRNA. To our knowledge these two assays have never been compared previously, so before we proceded to analyze the primary and secondary breast tumor samples for clone 4 mRNA expression by TP-PCR, it was necessary to compare the RNase protection assay with the TP-PCR assay, to facilitate any comparisons of the current results with those previously obtained.

Total RNA was extracted from frozen tissues from twenty five human breast tumors with ER values ranging from 0 to 311 fmol/mg, as determined by ligand binding assay. Ten micrograms of each RNA sample were analyzed in a standardized RNase protection assay in order to determine the absolute amount of C4 and WT-ER mRNAs within each sample. A typical autoradiogram of such an assay is shown in Figure 1. The signals corresponding to C4 and WT-ER mRNAs were quantified as described in the Materials and Methods section. In each assay, known amounts of synthetic WT-ER and C4 mRNAs were analyzed in parallel in order to establish a standard curve (Figure 2) allowing the determination of absolute levels of C4 and WT-ER mRNAs, expressed as pg/10 mg RNA (Table 1). Because of the very low C4 protected fragment signal ($\leq$ 15 dpm) in seven tumors, it was not possible to determine confidently the absolute amount of C4 mRNA in these samples (not determined, nd). All C4 negative tumors by RNase protection assay were from tumors with ER values lower than 10 fmol/mg, as determined by ligand binding assay. For the eighteen remaining samples, ER as determined by ligand binding assay values ranged from 1.2 to 311 fmol/mg. The absolute amounts of C4 and WT-type ER mRNAs were determined by RNase protection assay and varied from 2 to 83.9 pg/10 mg RNA and from 9 to 3651 pg/10 mg RNA, respectively. For each

sample, the C4 mRNA signal was expressed as a percentage of the wild type ER mRNA signal (Table 1).

Clone 4 mRNA relative expression was determined by TP-PCR within the same twenty five RNA samples. One microgram of total RNA extracted from each breast tumor sample was reverse-transcribed and an aliquot of the cDNA amplified by TP-PCR in the presence of dCTP [$\alpha$-$^{32}$P] as described in the Materials and Methods section. PCR products were separated on 6% acrylamide denaturating gels. A typical autoradiogram is shown Figure 3. Both clone 4 and wild type ER cDNAs signals were detected in all twenty five tumors studied, independent of their ER status as determined by ligand binding assay. It should be stressed that an additional band can be observed in most of the samples. This band has been identified after subcloning and sequencing to be a product of an exon 2 duplicated ER variant mRNA. The intensity of the signal obtained from this exon 2 duplicated ER band parallels that of the wild type ER band. Clone 4 and wild type ER signals were quantified as described in the Materials and Methods section. The signal corresponding to clone 4 was expressed as a percentage of the wild type ER signal. Table 1 presents the average of a least two independent TP-PCR experiments. Linear regression analysis (Figure 4) shows a highly significant correlation between clone 4 mRNA relative expression as determined by RNase protection assay (in the eigtheen tumors where a clone 4 signal was detectable) and clone 4 mRNA relative expression determined by TP-PCR ($r = 0.932$, $P < 0.0001$). This correlation between the RNase protection assay and TP-PCR results suggests that TP-PCR evaluation of the clone 4 mRNA relative to wild type ER mRNA expression is accurate and can be used to compare breast tissue samples. The co-amplification of the exon 2 duplicated ER variant mRNA using TP-PCR does

not seem to interfere with the relationship between TP-PCR and RNase protection assay.

Using the above TP-PCR assay, the median relative expression of the truncated cloned 4 ER for the primary tumors was 3.5 % (range1.6 - 10.5%) and the median value for the matched lymph node metastases was 3.1 % (range 1.0 - 19.4%). The scatter plot of these results is shown on Figure 5A. Using a Wilcoxon rank sum analysis, there is no statistically significant difference in the relative expression of clone 4 ER variant expression between primary breast tumors and their concurrent lymph node metastases.

Multiple ER variant mRNAs have been shown to be expressed in any one breast tissue sample (1, 7). In particular exon deleted ER variant mRNAs are frequently detected in breast tumors as well as other tissues (16). Therefore, to more fully investigate the pattern of ER variant expression between primary breast tumors and their matched lymph node metastases the relative levels of the exon 5 and exon 7 deleted ER variants were compared in the same 15 samples of primary human breast tumors and their matched lymph node metastases. The median relative expression of the exon 5 deleted ER for the primary tumors was 23.1 % (range17.3% - 94.3%) and the median value for the matched lymph node metastases was 31.3 % (range 14.9 % - 200%). The scatter plot for these results is shown in Figure 5B, and the difference is not statistically significant. The median relative expression of the exon 7 deleted ER for primary tumors was 65% (range 39.3% - 184.9%) and the median value for the matched lymph node metastases was 52.5% (range 35.5% - 126%). The scatter plot of these results is shown in Figure 5C and the difference is not statistically significant. Further using a longe range RT-PCR approach which allows the evaluation of the relative pattern of expression of all exon deleted ER variant transcripts present in any individual sample (13) we observed no

consistent change in the pattern of variant expression between any of the primary tumors or their matched lymph node metastases.

## DISCUSSION.

To our knowledge this study is the first that addresses the question of the comparison of an already established quantitative approach such as the RNase protection assay with an RT-PCR based approach in the study of ER variant mRNA expression. All of the studies published so far have either been done by RNase protection assay alone or by RT-PCR alone. Considering the potential clinical relevance of the measurement of the relative level of ER variants with respect to wild type ER within human breast tissue samples and the sensitivity of an RT-PCR based approach, such a comparative study was deemed necessary. Furthermore, our data provide validation for comparing previous data obtained using a non-amplification dependent RNase protection assay with the current data obtained using an amplification dependent TP-PCR assay.

The lack of sensitivity of the RNase protection assay for a subset of tumors with very low (<10 fmol/mg) ER values by ligand-binding assay is an important limiting factor. It effectively means that in a screening study, ER negative tumors (<3 fmol/mg protein) as well as ER positive tumors with ER values lower than 10 fmol/mg, as measured by ligand binding assay, cannot be reliably assessed for clone 4 ER variant mRNA expression by RNase protection assay. This together with the relatively large amount of RNA needed to perform an RNase protection analysis severely limits the usefulness of a standardized RNase protection assay in such screening studies. Our studies show that an already described TP-PCR technique is a more practical alternative to the RNase protection assay, especially when tissue sample size and therefore amount of extracted RNA is limiting. The low amount of starting material needed, together with the higher sensitivity observed (samples clone 4 ER

variant negative by RNase protection assay had detectable levels of clone 4 ER variant and wild type ER mRNA by TP-PCR) make TP-PCR an attractive alternative to the RNase protection assay in studies where such factors are limiting. It should be underlined that this TP-PCR approach can be adapted to all models where the relative quantification of two RNAs sharing one common sequence (initiated from different promoters, for example) is of importance.

It should be noted that because of the design of the riboprobe (covering wild type ER exon 2 sequence and clone 4 ER variant specific sequence) used in this and our previous study (9), the protected fragment from the exon 2 duplicated RNA would be included in the wild type ER signal using the RNase protection assay. In addition because the upstream primer in the TP-PCR is situated in exon 2, amplification of the exon 2 duplicated ER results in both wild type ER and exon 2 duplicated sized products. Similarly, because several exon deleted ER variants have been observed in human breast tissues (1), one should note that whether RNase protection assay or TP-PCR is used, the signal attributed to wild type ER mRNA corresponds to a signal representing all ER-like mRNA containing the exon 2 and exon 3 sequences. The values obtained using both techniques are therefore representative of the balance existing between clone 4 ER variant mRNA and ER-like mRNAs, including wild type ER but also exon7-deleted, exon 5-deleted, exon 4-7-deleted etc. mRNAs. Interestingly, correlation between relative measurements of the mRNA levels of wild type ER and clone 4 ER variant using RNase protection assay and TP-PCR are in better agreement with each other than is the measurement of wild type ER by RNase protection assay compared with the ligand-binding assay (Table 1). This may in part be due to tumor heterogeneity, i.e. the portion of the tumor used in the ligand-binding assay may have had a much different cellular composition (normal and neoplastic epithelial cells, normal stroma, myoepithelial cells) than

the portion used for RNA extraction. Another possible explanation is that the ligand-binding assay is in fact a measurement of an ER protein which contains a functional ligand-binding domain, and some tumors might be expressing higher levels of variant ERs which are lacking a hormone-binding domain. This hypothesis is supported by a recent study in which a subset of human breast tumors was identified which presented stronger signals using an antibody recognizing an N-terminal epitope of the ER than with an antibody recognizing a C-terminal epitope (3). These tumors were shown to express relatively higher levels of variant ER mRNAs, including clone 4 variant ER mRNA, which if translated would be missing the ligand-binding domain of the ER and would not be detected in a ligand-binding assay.

The data presented in this report provide evidence that both the pattern of ER variant expression and the relative level of expression of three individual ER variants are conserved in primary breast tumors and their matched, concurrent lymph node metastases. These findings are not inconsistent with our previously published data in which the relative expression of at least one ER variant was significiantly increased in primary tumors with poor prognostic characteristics, which included having concurrent lymph node metastases, as compared to primary tumors without concurrent lymph node metastases (9). The primary tumors in our current study by definition fall into the previously described poor prognostic group since they are all primary breast tumors with concurrent lymph node metastases. Further, the observations presented in this manuscript are consistent with previous observations that little change of ER status is found between primary human breast tumors and their concurrent lymph node metastases (17, 18). Since altered expression of several ER variants has been shown to occur in primary breast tumors compared to normal human breast tissues (6, 7), as well as between good versus poor prognosis

primary breast tumors, the current data suggest that alterations of ER variant expression and any role it has in altered estrogen signal transduction must occur before the acquisition of the ability to metatasize to other tissue sites. Furthermore, previous data support the concept of an early involvement of perturbations of estrogen signal transduction and the development of hormone independence in breast tumorigenesis (19, 20). While the exact role of ER variants in ER signal transduction is unclear, previous data together with the current data would be consistent with a role in the early events of tumorigenesis and breast cancer progression.

**ACKNOWLEDGEMENTS.**

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## LEGENDS TO FIGURES.

Figure 1: Quantification of C4 and WT-ER mRNAs by RNase protection assay

Ten micrograms of RNA extracted from twenty five breast tumor samples were analyzed as described in the Materials and Methods section. In each experiment, a synthetic RNA preparation (S1: 30 pg clone 4 mRNA, 125 pg wild type ER mRNA; S2: 125 pg clone 4 mRNA, 500 pg wild type ER mRNA; S3: 500 pg clone 4 mRNA, 2000 pg wild type ER mRNA) was analyzed in parallel to establish a standard curve. The bands migrating at 257 bases and 302 bases correspond to wild type ER and clone 4 mRNAs, respectively. Signals were counted and absolute amounts of clone 4 and wild type ER mRNA determined as indicated in the Materials and Methods section. The film presented was overexposed to allow the visualization of the clone 4 protected fragment in most of the samples.

Figure 2: Standard curve allowing the subsequent quantification of clone 4 and wild type ER mRNA and their relative expression within breast tumor samples by RNase protection assay

Signals corresponding to clone 4 and wild type ER mRNAs standard dilutions (S1, S2 and S3, Figure 2) were measured and a standard curve established. Signals measured for each sample were compared to this curve generated for each assay, in order to quantify clone 4 and wild type ER mRNAs in each tumor. Results are expressed as pg/10 mg of total RNA.

Figure 3: Determination of clone 4 mRNA relative expression by TP-PCR

For each tumor, 1 mg of RNA was reverse-transcribed before performing TP-PCR in the presence of dCTP [$\alpha$-$^{32}$P] as described in the Materials and Methods section. PCR products were separated by PAGE and corresponding signals

quantified as described in the Materials and Methods. The bands migrating at 148 bp and 536 bp correspond to wild type ER and clone 4 cDNAs, respectively. M: molecular size marker (Fx174, Gibco BRL, Grand Island, NY).

Figure 4: Linear regression analysis of clone 4 expression (expressed as a percentage of the corresponding WT-ER expression) as determined by TP-PCR versus standardized RNase protection assay in eighteen human breast tumors.

Figure 5:

A. Quantitative comparison of the relative expression of clone 4 variant ER mRNA in primary (1°) human breast tumors ( ) and their concurrent matched lymph node metastases (2°, ). For each sample the mean of three independent measures of clone 4 relative expression expressed as a percentage of the corresponding wild type ER signal was determined as described in the Materials and Methods section.

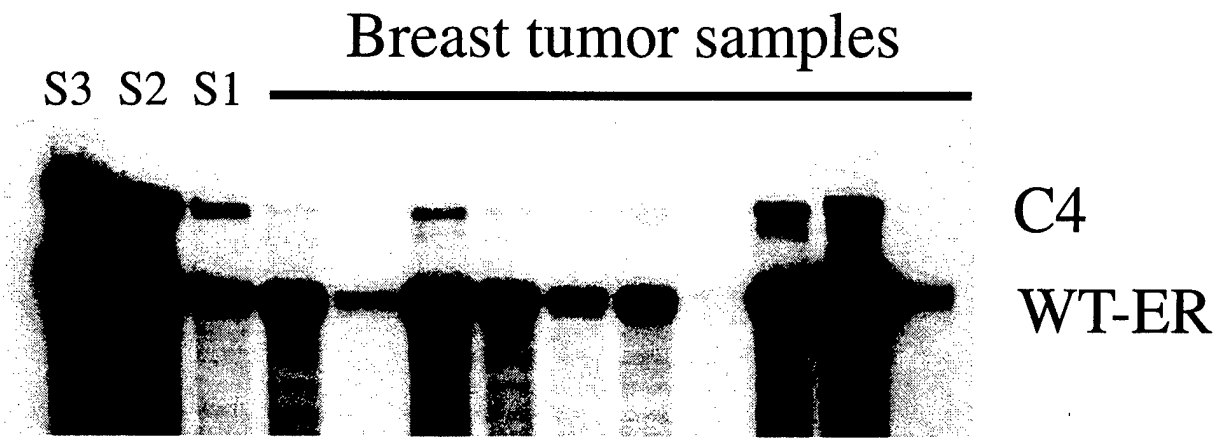
B. Quantitative comparison of the relative expression of exon 5 deleted variant ER mRNA in primary (1°) human breast tumors ( ) and their concurrent matched lymph node metastases (2°, ). For each sample the mean of three independent measures of exon 5 deleted ER relative expression expressed as a percentage of the corresponding wild type ER signal was determined as described in the Materials and Methods section.

C. Quantitative comparison of the relative expression of exon 7 deleted variant ER mRNA in primary (1°) human breast tumors ( ) and their concurrent matched lymph node metastases (2°, ). For each sample the mean of three independent measures of exon 7 deleted ER relative expression expressed as a percentage of the corresponding wild type ER signal was determined as described in the Materials and Methods section.

## Table legends

Table 1: C4 and WT-ER mRNA expression in twenty five human breast tumors, as determined by RNase protection assay and TP-PCR

Figure 1

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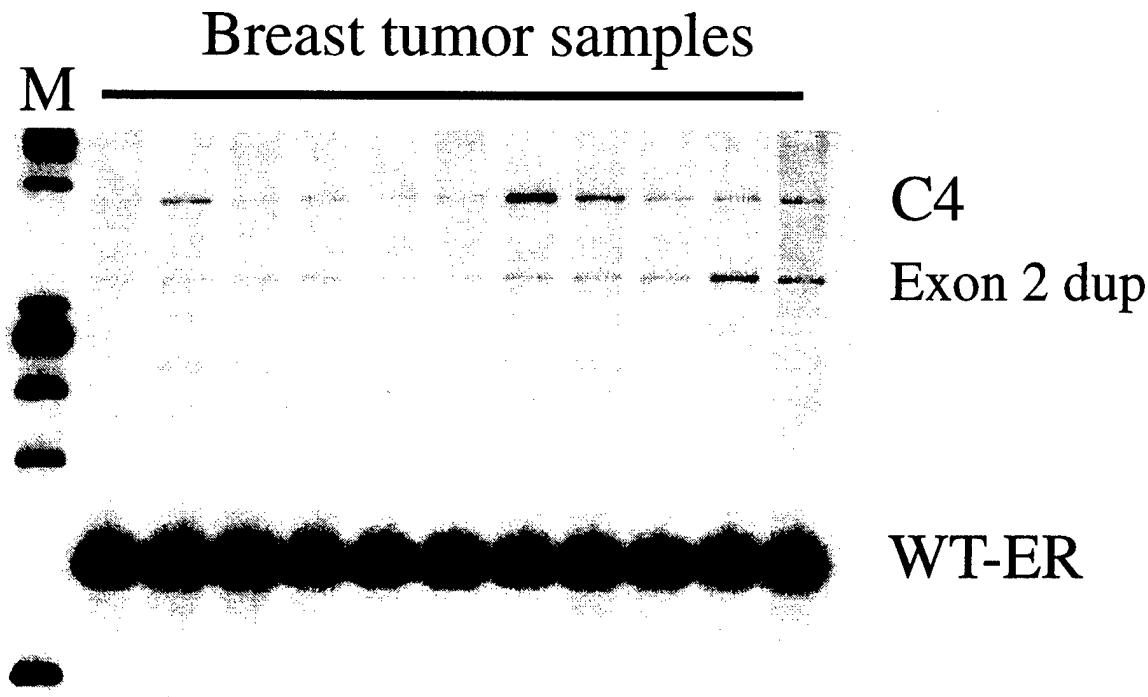

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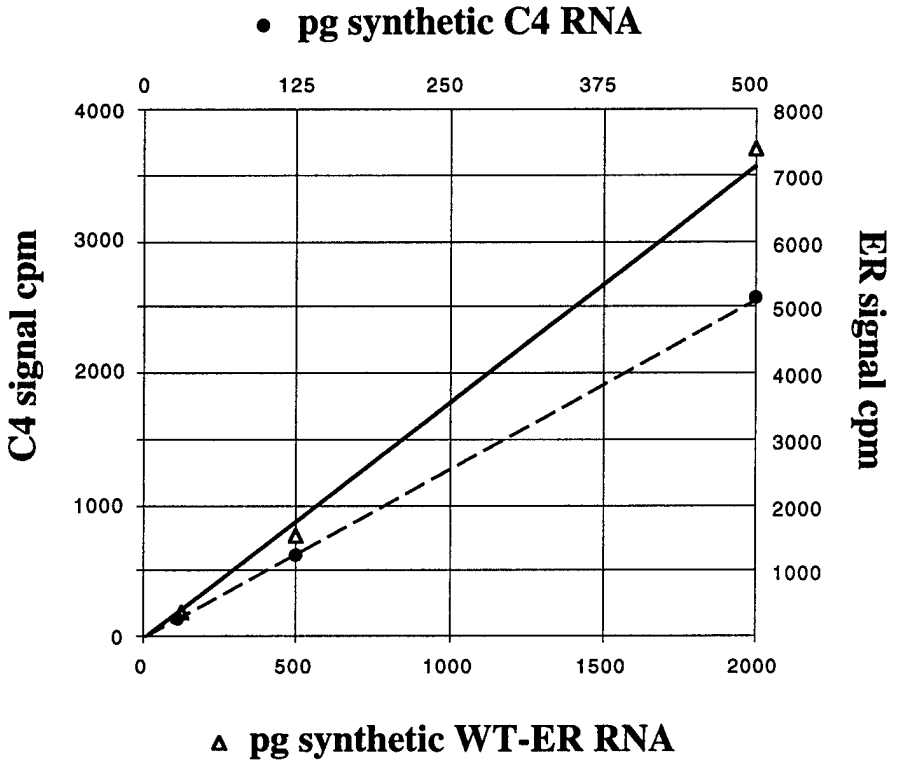
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Figure 4

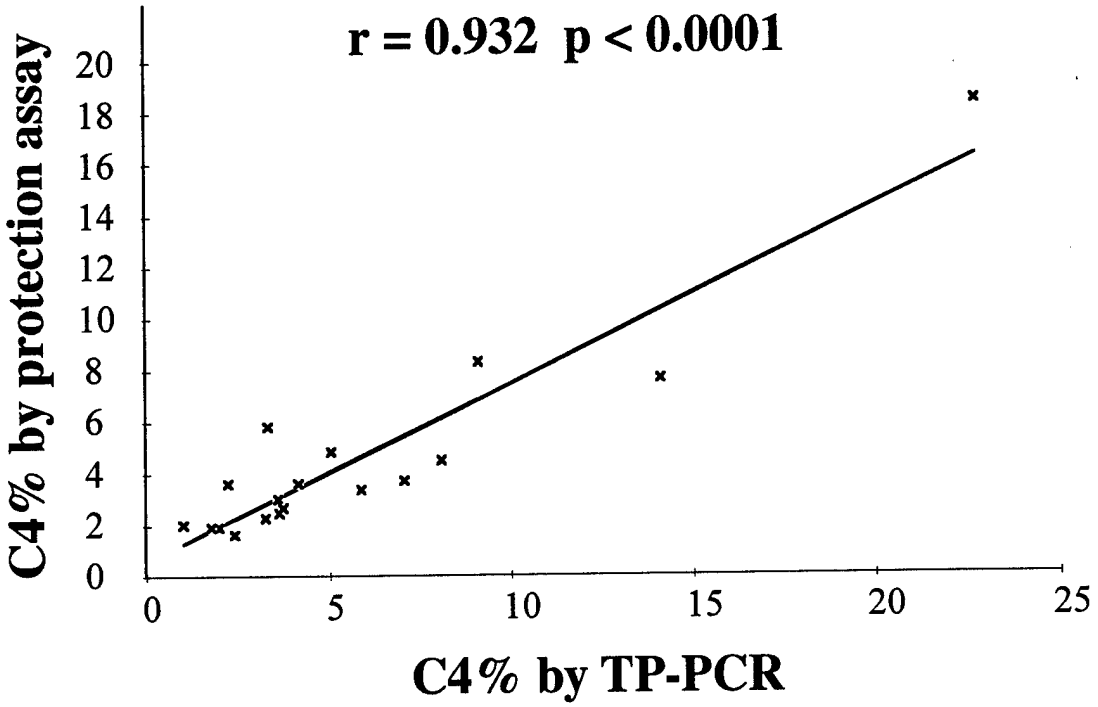
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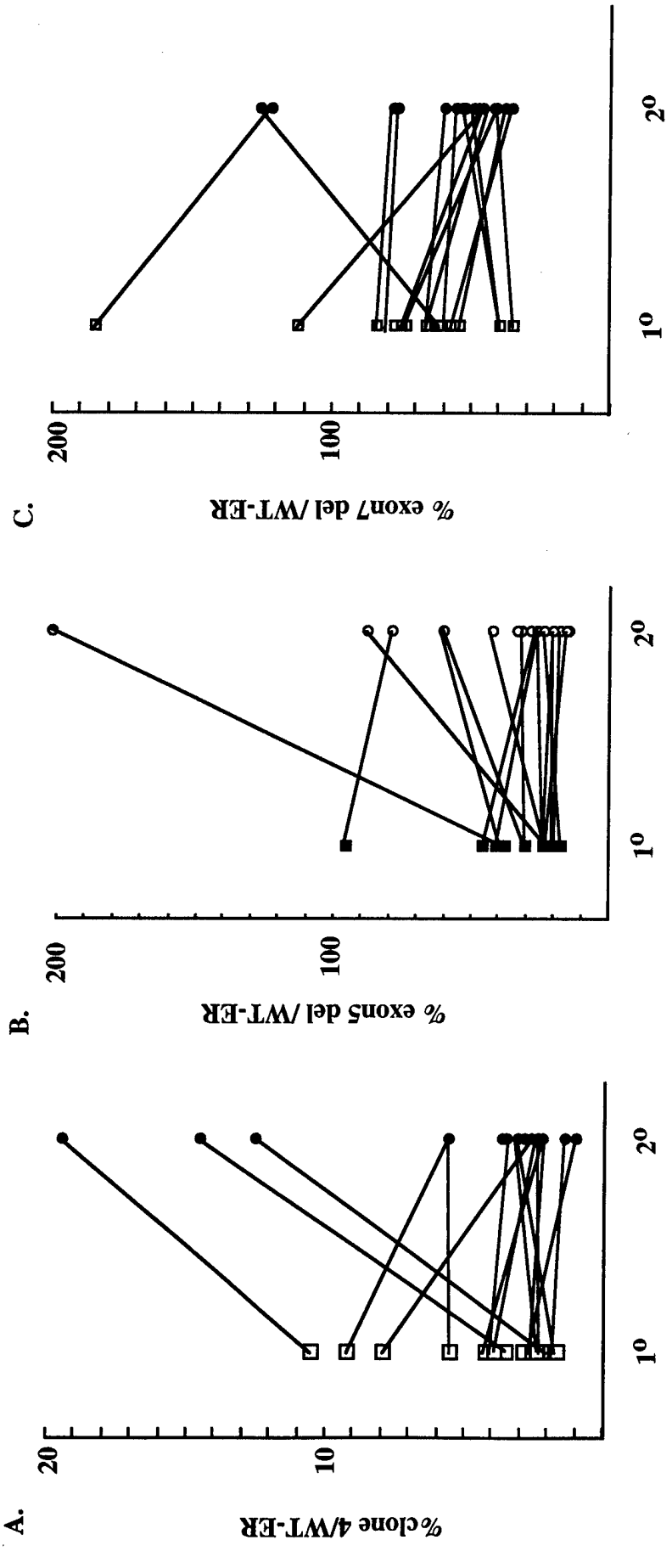
Figure 5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2⁰
C.
% exon7 del /WT-ER
200
100
1⁰
2⁰

| Sample N° | Ligand Binding | RNase protection | | | TPPCR |
|---|---|---|---|---|---|
| | ER fmole mg | C4 pg/10µg | WT-ER pg/10µg | C4 % | C4 % |
| 5 | 0.0 | nd[a] | nd | - | 1.7 |
| 3 | 0.4 | nd | nd | - | 2.6 |
| 1 | 0.9 | nd | nd | - | 3.1 |
| 24 | 1.2 | 6.2 | 105.1 | 5.9 | 3.3 |
| 4 | 1.8 | nd | nd | - | 3.7 |
| 23 | 4.5 | 10.0 | 54.3 | 18.4 | 22.7 |
| 8 | 5.8 | nd | 26.8 | - | 2.8 |
| 7 | 6.3 | nd | 224.6 | - | 3.4 |
| 2 | 8.7 | nd | 9.0 | - | 2.2 |
| 19 | 10.0 | 22.6 | 902.9 | 2.5 | 3.6 |
| 10 | 17.8 | 5.3 | 146.4 | 3.6 | 4.1 |
| 13 | 25.0 | 2.3 | 112.0 | 2.0 | 1.0 |
| 15 | 44.0 | 5.0 | 148.5 | 3.4 | 5.9 |
| 22 | 57.0 | 11.8 | 153.6 | 7.7 | 14.1 |
| 11 | 90.0 | 2.5 | 129.1 | 1.9 | 1.7 |
| 21 | 96.0 | 9.6 | 263.4 | 3.6 | 2.2 |
| 14 | 105.0 | 4.6 | 94.4 | 4.9 | 5.0 |
| 17 | 111.0 | 26.7 | 320.3 | 8.3 | 9.1 |
| 9 | 121.0 | 4.6 | 277.7 | 1.7 | 2.4 |
| 6 | 146.0 | 2.0 | 105.0 | 1.9 | 1.9 |
| 18 | 198.0 | 15.8 | 422.0 | 3.7 | 7.0 |
| 20 | 236.0 | 8.8 | 288.4 | 3.0 | 3.5 |
| 12 | 289.0 | 3.6 | 80.5 | 4.5 | 8.0 |
| 16 | 304.0 | 38.8 | 1440.8 | 2.7 | 3.7 |
| 25 | 311.0 | 83.9 | 3651.0 | 2.3 | 3.2 |

# APPENDIX 7

Breast tumor cells had marginally detectable transcripts, benign breast cells had transcripts if detectable initiating from PEII primarily and PEI.3. In both treatment with dexamethasone (D), phorbol ester (PDA) and cAMP increased transcripts substantially: by D using PEI.4, PCA+cAMP using PEII, and D+PCA+cAMP using PEI.4 and PEII. PEI.1/PEI.2/PEI.2a use was not detectable in any breast samples. Therefore, in breast myofibroblasts both glucocorticoid receptor, and intracellular signalling molecule (cAMP and PDA) activation of protein kinase, mechanisms are implicated in transcriptional regulation of aromatase. (Supported by NCI RO1 CA65622)

**#1984 Loss of melatonin is not a significant contributor to MCF-7 and BG-1 cell proliferation.** Baldwin, W.S., Risinger, J.I., and Barrett, J.C. *National Institute of Environmental Health Sciences, RTP, NC 27709*

Epidemiological evidence suggests that electromagnetic fields (EMF) induce a variety of cancers, including breast cancer. It has been postulated that EMF induces breast cancer due to the inhibitory effects EMF has upon melatonin production. Melatonin may act through several proposed mechanisms to inhibit breast and other cancers. We examined two of these mechanisms in vitro. Does melatonin protect cells from oxygen radicals and does it attenuate estrogen induced cell growth? Melatonin protected MCF-7 cells from $H_2O_2$-induced death at 10 $\mu$M concentrations, but not at physiological concentrations. Next, we examined melatonin's effects upon estradiol-induced proliferation in MCF-7 and BG-1 cells. Melatonin attenuated proliferation during basal growth, but not following estradiol exposure. This work was also repeated with estrogen starved cells to mimic in vivo conditions with no difference in results. Further work demonstrated that melatonin could not inhibit estradiol-induced G1 to S phase cell cycle transition, nor could it inhibit estradiol-induced PS2 mRNA levels. Both proliferation studies and the use of biomarkers demonstrated that melatonin does not inhibit estradiol-induced proliferation, suggesting that EMF-induced loss of melatonin is not important in the etiology of breast cancer. However melatonin homeostasis may have other effects upon estradiol production that were not addressed in this study.

**#1985 Progestins do not stimulate mammary gland proliferation in a postmenopausal animal model.** Raafat, A., Li, S., and Haslam, S.Z. *Physiology Dept., Michigan State University, E. Lansing, MI 48824*

Estrogen (E) plus progestin (P) are believed to cause maximal stimulation of mammary cell proliferation in the rodent and human mammary glands. While combined hormone replacement therapy (CHRT) with E+P is commonly prescribed for postmenopausal women, its effects on mammary gland proliferation and potential impact on breast cancer risk are poorly defined. The purpose of the present study was to determine the effect of E+P on mammary cell proliferation in a postmenopausal mouse model. We found that in postmenopausal mice, the stimulatory effect of E+P was the same as E alone. In contrast, E+P increased cell proliferation significantly more than E alone only in non-menopausal controls. These differences were also reflected in changes in mammary gland morphology, with ductal sidebranching observed only in E+P-treated non-menopausal controls. Analysis of E regulation of mammary PR revealed that PR levels could be increased only in the non-menopausal controls. The lack of mammary PR regulation by E in postmenopausal mice was tissue specific since E increased uterine PR to the same extent in both postmenopausal and control mice. Thus, low PR levels and absence of PR regulation by E likely explain the lack of increased mammary proliferation upon E+P treatment in postmenopausal mice. These results suggest that the addition of P in CHRT may not increase mammary proliferation or impact on breast cancer risk over that observed with E alone. Supported by NIH grant R01AG 13059.

**#1986 Role of estrogen receptor in enhanced estrogen-induced mammary gland proliferation in a postmenopausal animal model.** Raafat, A., Li, S., Bennett, J., and Haslam, S.Z. *Physiology Dept., Michigan State University, E. Lansing, MI 48824*

Conflicting epidemiological evidence on postmenopausal hormone replacement therapy (HRT) and increased breast cancer risk indicates that a direct analysis of HRT on mammary tissue is needed. Using ovariectomy-induced menopause in mice as a model, we have found that estrogen (E) caused 2-fold increased proliferation in mammary tissue over non-menopausal controls. The present studies were undertaken to determine if the enhanced response is due to increased E efficacy or potency. Dose response studies showed that increased E efficacy is the basis for the enhanced proliferation. To define the role of estrogen receptors (ER), ER concentration and cellular distribution were analyzed immunohistochemically. While there was no difference in the number of ER positive epithelial cells, there were 25% more ER positive stromal cells in postmenopausal glands. Furthermore, there was a significant (26%) reduction in E-induced ER down regulation in the epithelium of postmenopausal glands. Thus the higher ER content in stromal cells together with the greater persistence of ER in epithelial cells most likely contribute to the increased proliferation. These results suggest that in the postmenopausal gland, increased ER content and altered regulation could result in a significant proliferative response to HRT and possible increased breast cancer risk. Supported by NIH Grant ROIAG13059

**#1987 Activation of 17$\beta$-estradiol and estrone by dimethyldioxirane and the transcriptional effects on DNAs with known base content and sequence.** Yu, F., Bender, W., Weber, R., Ayyagari, S., Suchobrus, H., Hagshenas, L., Mallia, A., and Mallia, P. *University of Illinois Coll. of Med., Rockford, IL 61107*

Estrogens, used widely from hormone replacement therapy to cancer treatment, are themselves carcinogenic, causing uterine, liver and breast cancers. The mechanism is not understood. We found that estrone(E1), 17$\beta$-estradiol(E2), diethylstilbestrol(DES) and tamoxifen(TAM) can be activated by the epoxide-forming oxidant, dimethyldioxirane(DMDO) and to inhibit rat liver nuclear and nucleolar RNA synthesis *in vitro*. A hypothesis is proposed suggesting epoxidation of estrogens is the underlying mechanism of carcinogenesis (Carcinogenesis 17, 1957–1961,1996). This reports the transcriptional effects of the DMDO activated E1, E2, DES and TAM on poly[d(I-C)], poly[d(A-T)], polydG·polydC, and polydC. The results show that there are differences in the degrees and specificities of inhibition. E1 and E2 show similar inhibition toward poly[d(I-C)], poly[d(A-T)], polydG·polydC and has little effect on polydC. DES has a very strong inhibition on polydC. TAM is only able to produce significant inhibition on polydG·polydC template. (Summer high school research fellows R.W., S.A., H.S., L.H., A.M., P.M.; supported by NCI CA-70466).

**#1988 Mechanisms of hormone-independence in human breast cancer cells.** Coutts, A.S., Leygue, E., and Murphy, L.C. *University of Manitoba, Wpg, MB, Canada R3C 2E3*

The evolution of breast cancer into an estrogen-independent growth phenotype marks the beginning of a more aggressive phase of the disease and is a major problem in the efficacy of endocrine therapies. In some cases, hormone-independence and resistance can occur due to loss of estrogen receptor (ER) expression, but at least 50% of tumors which have developed resistance to endocrine therapy remain receptor positive. T-47D5 human breast cancer cells are ER+ and estrogen treatment in culture results in increased proliferation of these cells. An estrogen-nonresponsive cell line (T5-PRF) was developed from T-47D5 cells, by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen while still retaining expression of the ER. Transient transfection studies have been performed using an estrogen-responsive reporter gene system. In the absence of ligand T5-PRF cells have ~4 fold increased basal ER activity compared to the parent T-47D5 cells. Long range ER RT-PCR has also been performed to characterize the pattern of variant ER mRNA expression between the two cell lines and differential expression of ER mRNA variants was found. In particular, an ER variant mRNA, deleted in exons 3 and 4, was detected only in T5-PRF cells. These results suggest that defects in the ER structure and function, or activation of ER via ligand-independent mechanisms may underly hormone-independence in breast tumors.

**#1989 Steroid receptors in breast cancer patients in Kuwait.** Luqmani, Y.A., Temmim, L., Memon, A., Ali, M.A.A., and Parkar, A.H. *Kuwait Cancer Control Center [Y.A.L., L.T., A.M., M.A.A.A., A.H.P.], Kuwait University [Y.A.L., A.M.]*

Estrogen and progesterone receptors (ER, PR) were measured in cytoplasmic and nuclear extracts of breast cancer tissue from 799 patients, by ligand binding assay (LBA) or enzyme immunoassay (EIA). Receptor levels were much lower than widely reported in the literature. Frequency of positivity, using consensus cut off values, was lower than reported by the EORTC Group. Measurements by the two methods were statistically correlated, in terms of positivity based on criteria for clinical assessment, but concordance was poor, particularly for ER assayed in the same samples by the two methods. In cytosolic but not nuclear extracts, LBA gave higher median values for ER than EIA; for PR they were higher with EIA in both cell fractions. Correlation was excellent between receptor levels in cytosolic and nuclear extracts for both ER and PR using EIA; significantly better than with LBA. There was a correlation between ER and PR in both cytosolic and nuclear fractions particularly when analysis was by EIA. ER in the cytosolic fraction also correlated with PR in the nuclear fraction and ER in the nuclear fraction with PR in the cytosolic fraction, but only with EIA. Disagreement between the methods may be legitimately due to presence of receptor isoforms, which may have biological significance. Though presence of receptor in the cytosolic fraction is artifactual, it's measurement by EIA parallels the level of receptor in the nuclear fraction.

**#1990 Involvement of the transcription factor, E2F, in the biphasic response of human breast tumor cells to estradiol.** Jain, P.T., and Gewirtz, D.A. *Departments of Pharmacology/Toxicology and Medicine, Medical College of Virginia, Box 230, Richmond, VA 23298*

The transcription factor, E2F, which is regulated by both Rb and $p21^{waf1/cip1}$, influences the G1 to S transition via its binding to the promoter regions of genes which control DNA synthesis, including c-*myc*. It is proposed that both the stimulation of breast tumor cell growth by physiological concentrations of estradiol (0.1–10 nM) and the inhibition of growth by pharmacological concentrations of estradiol (1–100 $\mu$M) could involve regulation of c-*myc* expression through the transcription factor, E2F. Using an E2F binding site - luciferase reporter construct transiently transfected into MCF-7 breast tumor cells, we assessed the influence of estradiol on E2F activity. 100 $\mu$M estradiol (which inhibits growth) reduced E2F activity by 45% while 10 nM estradiol (which stimulates growth) enhanced E2F activity by approximately 50%. In MCF-7 cells transiently transfected with an

# APPENDIX 8

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# Expression of Estrogen Receptor Variant Messenger RNAs and Determination of Estrogen Receptor Status in Human Breast Cancer

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***Estrogen receptor (ER) status of breast cancer can be assessed by immunohistochemical assay (IHA), although we have previously observed that ER-IHA levels can be inconsistent between amino-terminal and carboxyl-terminal-targeted antibodies. To address the hypothesis that this discrepancy is attributable to expression of ER variant mRNAs encoding truncated ER-like proteins, we have studied 39 IHA-consistent and 24 IHA-inconsistent breast tumors by reverse transcription polymerase chain reaction to examine the expression of multiple exon-deleted (D-ER) variant mRNAs and the truncated ER clone 4 variant mRNA. ER variants D7-ER, D4-ER, D3–4-ER, and D4–7-ER were detected at similar frequencies in both groups. However, ER variants D2–3/7-ER, D2–3-4-ER ($P < 0.05$), and D-3–7-ER ($P < 0.01$), which encode putative short ER-like proteins that might be recognized only by an amino-terminal-targeted antibody, were preferentially detected in inconsistent cases. ER clone 4 mRNA expression was also higher in inconsistent tumors ($P < 0.001$). Further analysis showed that, whereas overall prevalence of ER variant mRNAs was similar in both tumor groups, occurrence of the subset of variant mRNAs encoding putative truncated proteins was also higher in IHA-inconsistent tumors ($P < 0.05$). These data suggest that ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER-IHA levels determined using amino- or carboxyl-terminal-targeted antibodies. (Am J Pathol 1997, 150:1827–1833)***

Estrogen receptor (ER) determination is an important parameter in the clinical management of breast cancer.[1,2] Until recently, ER content was assessed principally by ligand-binding techniques such as dextran-coated charcoal (DCC) or sucrose gradient assays. Now, with the development of several antibodies able to recognize ER protein, immunohistochemical assay (IHA) has become an alternative approach to determine ER status of breast tumors and to predict endocrine response in breast cancer.[3,4] The ER-IHA approach has significant advantages including the potential for parallel assessment of tumor cell content and heterogeneity of ER expression. However it differs from traditional methods in that ER activity is defined by structural rather than functional criteria.

ER-IHA in tissue sections has been successfully achieved by several different antibodies, including 1D5, H222, and AER311, which are able to recognize different epitopes within particular domains of the ER protein (Figure 1).[3–5] However, we and others have previously observed that the ER-IHA results from some tumors are discordant between different antibodies that are able to recognize either the $NH_2$ or the COOH terminals, with a tendency to higher signals with $NH_2$-terminal-targeting antibodies.[4,5] Although these differences might relate to different antibody affinities, another explanation lies in the existence of ER variants. Beside the wild-type ER mRNA transcript, several ER variant mRNAs have been described in both normal and cancer tis-

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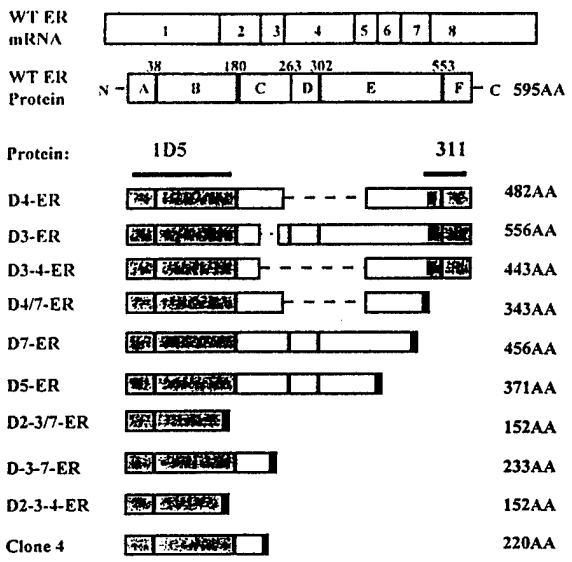


Figure 1. *Schematic presentation of WT-ER protein and the predicted proteins encoded by ER variant mRNAs. ER protein contains A to F functional domains. Region A/B of the receptor is implicated in transactivating function (TAF1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another transactivating function (TAF2). WT-ER reading frame is conserved in ER variant mRNAs deleted in exon 4, in exon 3, and in both exons 3 and 4. Encoded proteins from D4-ER, D3-ER, and D3–4-ER, respectively, are similar to WT-ER* (open box) *but miss some internal amino acids (- - -). Simple deletion of exon 7 or exon 5 and multiple deletion of exon 4 and exon 7; exons 2, 3, and 7; exons 3 and 7; and exons 2, 3, and 4 introduce a shift in the ER-WT reading frame. The resulting proteins, D7-ER, D5-ER, D4/7-ER, D2–3/7-ER, D-3–7-ER, and D2–3-4-ER, respectively, are therefore similar to WT-ER* (open box) *but are truncated of the C-terminal WT region* (black box, *indicating amino acids different from WT-ER). Clone 4 protein is encoded by an ER variant mRNA containing WT-ER exon 1 and exon 2 juxtaposed with line-1-related sequences. Clone 4 protein is similar to WT-ER* (open box ) *but is missing the C terminal. The gray areas represent regions of the protein that are theoretically recognized by 1D5 or AER311 antibodies.*

sues.[6–14] Most of these variants are suspected to result from alternative splicing of WT-ER mRNA and consist of exon-deleted and truncated variants.[6,8] Figure 1 shows some of the putative proteins encoded by these variants and illustrates that, whereas some of these altered proteins may still possess both $NH_2$- and COOH-terminal epitopes of the wild-type (WT) protein, others will be truncated and lack the COOH terminal as a result of an exon deletion that introduces a shift in the reading frame. In addition to exon-deleted ER mRNA variants, several truncated variants have been described, among which the ER clone 4 variant is highly prevalent in breast tumors.[8] The sequence of this variant mRNA corresponds to WT-ER exon 1 and 2 juxtaposed to line-1-related sequences, and *in vitro* analysis shows that it encodes a putative ER-like protein missing the carboxyl-terminal extremity.

To address the hypothesis that discrepancies observed by IHA using 1D5 and AER311 antibodies in breast tumors could result from particular ER variant expression, we investigated 39 IHA-consistent and 24 IHA-inconsistent breast tumors for the most prevalent exon-deleted ER variant mRNAs and in parallel for the level of ER clone 4 truncated variant mRNA expression by two reverse transcriptase polymerase chain reaction assays that we have recently developed to assess multiple ER variants in breast cancer tissues.[15,16]

## Materials and Methods

### Human Breast Tissues and ER Status Determination

The study was carried out on 63 cases of invasive ductal and invasive lobular breast carcinomas obtained from the NCIC-Manitoba Breast Tumor Bank.[17] These cases correspond to the ER-positive subset of a series of 97 tumors previously studied by IHA.[5] In all cases, the specimens had been rapidly frozen at −70°C as soon as possible after surgical removal. Subsequently, a portion of the frozen tissue from each case was processed routinely to create formalin-fixed, paraffin-embedded tissue blocks that were matched and orientated relative to a corresponding frozen tissue block. Paraffin sections were previously analyzed by IHA using 1D5 (Dako, Dimension Labs, Mississauga, Canada) and AER311 (Neomarkers, Lab. Vision Corp., Fremont, CA) ER monoclonal antibodies.[5] In each case, immunohistochemical staining was assessed, without knowledge of the ER DCC status or antibody used, by a semiquantitative H score system (range, 0 to 300) for both antibodies and in the same regions on adjacent serial sections. When a difference of H score values between the two antibodies was >50, tumors were classified as inconsistent. When the difference of H score values was <50, the tumors were considered as consistent. Overall, the mean ER and progesterone receptor (PR) status and the distribution of ER and PR levels between the inconsistent and consistent groups was very similar (see Table 1). Within the inconsistent tumor group (24 cases), 8 tumors were low ER positive (3 to 10 fmol/mg protein; 33%), 6 tumors were middle ER positive (11 to 50 fmol/mg protein; 25%), and 10 were high ER positive (>50 fmol/mg protein; 42%), as determined by ligand-binding assay. Within the consistent tumor group (39 cases), 6 cases were low ER positive (15%), 12 cases were middle ER positive (31%), and 21 were high ER positive (54%).

Table 1. *Number of Tumors Expressing Detectable ER Variant in Consistent and Inconsistent Tumors*

| Tumors | n | $ER^{DCC}$ | $PR^{DCC}$ | D7-ER | D4-ER | D3-4-ER | D4/7-ER | D2-3/7-ER | D2-3-4-ER | D-3-7-ER | ER $V.^{OF}$ | ER $V.^{IF}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consistent | 39 | 81 (89) | 60 (75) | 35 | 9 | 3 | 4 | 0 | 0 | 2 | 6 | 12 |
| Inconsistent | 24 | 69 (86) | 55 (69) | 22 | 6 | 2 | 2 | 2 | 3 | 8 | 10 | 8 |
| P | | | | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | <0.05 | <0.01 | <0.05 | >0.05 |

Analysis was by $\chi^2$. ER $V.^{OF}$, out-of-frame exon-deleted ER variant mRNAs excluding D7-ER; ER $V.^{IF}$, in-frame exon-deleted ER variant mRNAs; $ER^{DCC}$, mean (SD) ER status measured by DCC assay (fmol/mg protein); $PR^{DCC}$, mean (SD) PR status measured by DCC assay (fmol/mg protein).

### *Extraction of mRNA and Reverse Transcription*

For each case, a specific face of a frozen tissue block that matched the corresponding face of the paraffin block previously studied by IHA was selected.[17] Total RNA was extracted from histologically defined regions within 20-$\mu$m cryostat sections of frozen tissue using a small-scale RNA extraction protocol (Trireagent, MRCI, Cincinnati, OH) as previously described.[18] Reverse transcription reactions were performed in triplicate in a final volume of 15 $\mu$l,[13,15] and 1 $\mu$l of the reaction mixture was taken for subsequent PCR amplification in either long-range PCR or triple-primer PCR assays described below.

### *Analysis of Prevalence of ER Variant mRNAs*

Prevalence of ER variant mRNAs within breast tumor samples was assessed by PCR analysis performed by a long-range PCR assay as previously described.[15] The primers used consisted of 1/8U primer (5′-TGCCCTACTACCTGGAGAACG-3′, sense, located in WT-ER exon 1) and 1/8L primer (5′-GCCTCCCCCGTGATGTAA-3′, antisense, located in WT-ER exon 8). This primer set allowed amplification of a 1381-bp fragment corresponding to WT-ER mRNA and all deleted or inserted ER variant mRNAs containing exon 1 and exon 8 sequences. PCR amplifications were performed in a final volume of 10 $\mu$l, in the presence of 10 nmol/L [$\alpha$-$^{32}$P]dCTP (ICN Pharmaceuticals, Irvine, CA), 4 ng/$\mu$l each primer, and 1 U of *Taq* DNA polymerase (Promega, Madison, WI). Each PCR consisted of 40 cycles (1 minute at 60°C, 2 minutes at 72°C, and 1 minute at 94°C) using a thermal cycler (MJ Research PT100, Fisher Scientific, Ottawa, Canada). After PCR, 2 $\mu$l of the reaction was denaturated in 80% formamide buffer, and the PCR products were separated on 3.5% polyacrylamide gels containing 7 mol/L urea (PAGE). After electrophoresis, the gels were dried and autoradiographed for 18 hours. Identities of specific bands were then confirmed by reference to size markers, subcloning, and sequencing.[13]

### *Quantification of ER Clone 4 mRNA Expression*

Quantification of clone 4 mRNA expression was performed using a triple-primer PCR assay as previously reported.[16] Briefly, three primers, E2U (5′-AGGGTGGCAGAGAAAGAT-3′, sense, located in WT-ER exon 2), E3L (5′-TCATCATTCCCACTTCGT-3′, antisense, located in WT-ER exon 3), and C4L (5′-GGCTCTGTTCTGTTCCATT-3′, antisense), were used during PCR, performed in the presence of [$\alpha$-$^{32}$P]dCTP. These primers allowed the co-amplification of a 281-bp and a 249-bp fragment corresponding to WT-ER and clone 4 truncated ER variant mRNAs, respectively. PCR products were separated by PAGE. After electrophoresis, gels were dried and autoradiographed. Autoradiographs were analyzed with a video-densitometry system and quantitated using MCID M4 software (Imaging Research, St. Catherines, Canada). The signal corresponding to ER clone 4 was measured relative to expression of the corresponding WT-ER and expressed as a percentage relative to a reference standard (an ER-positive tumor sample) to reduce any variation due to signal intensity in different gels. ER clone 4 expression was determined from the mean of three independent RT-PCR assays performed without knowledge of the IHA status. Means obtained from the 24 IHA-inconsistent tumor samples were then compared with those found in the 39 IHA-consistent tumor samples using the Mann-Whitney rank sum test (two sided).

## *Results*

### *Detection of Exon-Deleted ER Variant mRNAs within Consistent and Inconsistent Tumors*

Prevalence of exon-deleted ER variant mRNAs was investigated within 63 breast tumors, previously studied by IHA using 1D5 and AER311 antibodies[5] and subsequently classified as IHA consistent (39 cases) or IHA inconsistent (24 cases) as illustrated in

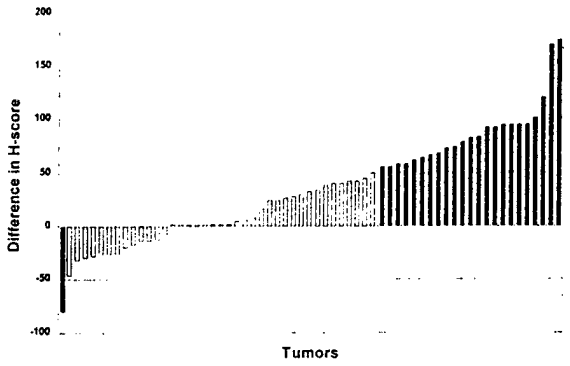


Figure 2. *Graph to show the difference in H score (IHA-1D5 to IHA-311) for each of 63 tumors showing the basis for classification into IHA consistent (< 50 H score difference:* white bars*) and IHA inconsistent tumors (> 50 H score difference:* black bars*).*

Figure 2. Long-range RT-PCR assay using primers annealing with exon 1 (1/8U) and exon 8 (1/8L) sequences first allowed assessment of the most prevalent exon-deleted variant mRNAs in comparison with the co-amplified WT-ER mRNA, as described previously.[15] Several different PCR products were observed within the set of tumors studied (Figure 3) that have previously been shown to correspond to the WT-ER (1381 bp) and ER variant mRNAs deleted in exon 7 (D7-ER, 1197 bp), exon 4 (D4-ER, 1045 bp), both exons 3 and 4 (D3–4-ER, 928 bp), exons 2, 3, and 7 (D2–3/7-ER, 889 bp), both exons 4 and 7

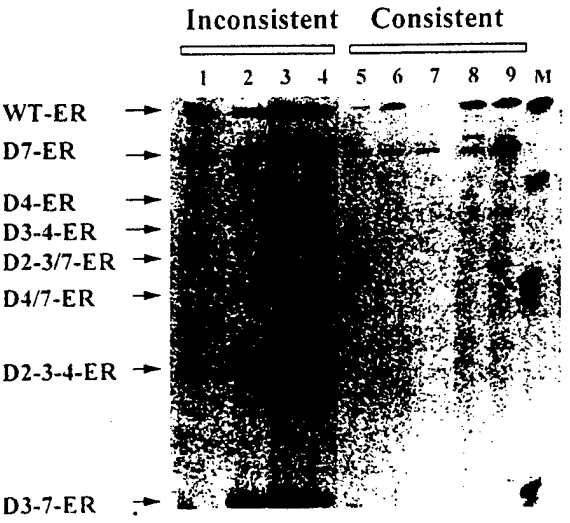


Figure 3. *Comparison of exon-deleted ER variant expression between IHA-consistent[1–4] and IHA-inconsistent[5–9] breast tumors. Total RNA was extracted from inconsistent and consistent tumors, reverse transcribed, and subsequently amplified by PCR as described in Materials and Methods. PCR products were separated on PAGE and visualized by autoradiography. Bands migrating at 1,381 bp, 1197 bp, 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp, and 580 bp were identified by isolation and sequencing as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3–4-ER), exons 2, 3, and 7 (D2–3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2, 3, and 4 (D2–3-4-ER), and within exon 3 to within exon 7 (D-3–7-ER), respectively. M, molecular weight marker ϕX174, Gibco BRL, Grand Island, NY.*

(D4/7-ER, 861 bp), exons 2, 3, and 4 (D2–3-4-ER, 737 bp), and within exon 3 to within exon 7 (D-3–7-ER, 580 bp), respectively.[15] Results obtained for IHA-consistent and IHA-inconsistent tumor subgroups are summarized in Table 1. D7-ER, D4-ER, D3–4-ER, and D4/7-ER variant mRNAs were detected at the same frequency in both subgroups. However, D2–3/7-ER, D2–3-4-ER, and D-3–7-ER mRNAs were preferentially detected in IHA-inconsistent tumors. This increased prevalence reached statistical significance for both D2–3-4-ER and D-3–7-ER mRNAs ($P < 0.05$ and $P < 0.01$). Given that the D7-ER variant was detected uniformly (>90%) in both subgroups, we chose to assess the remainder of the variant mRNAs that were not uniformly detected (ie, all variants except D7-ER). These were then considered with respect to the putative ER-like protein they should encode and classified further into two subgroups. In-frame variants (ER V.$^{IF}$) comprised those with a sequence modification that did not introduce a shift in the reading frame and that could encode proteins theoretically recognized by both 1D5 and AER-311 antibodies (D4-ER and D3–4-ER variant mRNAs). Out-of-frame variants (ER V.$^{OF}$) comprised variants encoding proteins theoretically only recognized by 1D5 antibody (D4/7-ER, D2–3/7-ER, D2–3-4-ER, and D-3–7-ER). ER V.$^{IF}$ were detected in 12 (31%) and 8 (33%) IHA-consistent and IHA-inconsistent tumors, respectively. At the same time, ER V.$^{OF}$ were detected in only 6 (15%) IHA-consistent compared with 10 (42%) IHA-inconsistent tumors, respectively ($P < 0.05$, $\chi^2$ analysis).

## *Quantification of Clone 4 mRNA Expression*

Expression of a prevalent truncated ER mRNA variant, the ER-clone 4 variant, which is also suspected to encode a truncated ER-like protein, was then analyzed by triple-primer RT-PCR using three primers to allow the co-amplification of WT-ER mRNA together with clone 4 variant mRNA, as described previously.[16] Typical results from IHA-consistent and IHA-inconsistent tumors are shown Figure 4. PCR products (bands of 281 bp and 249 bp) corresponding to WT-ER and ER clone 4 mRNAs were observed in all tumors. Using the Mann-Whitney rank sum test (two sided), the relative expression of clone 4 truncated variant ER mRNA to WT-ER mRNA was also found to be significantly ($P < 0.01$) higher in IHA-inconsistent tumors (median = 80.4%, SD = 18.7%) *versus* IHA-consistent tumors (median = 62.4%, SD = 14.4%; Figure 5)

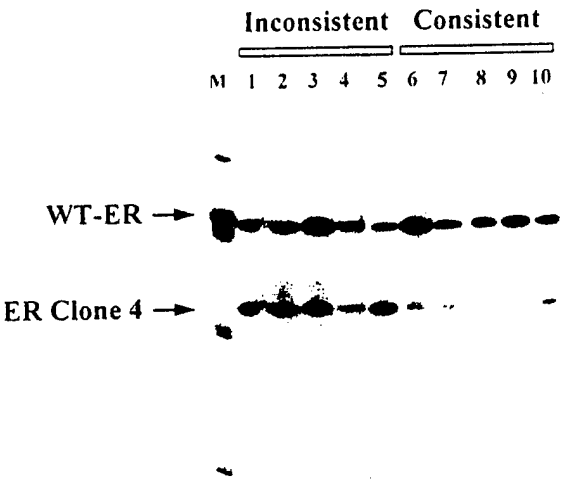


Figure 4. *Expression of clone 4 variant ER mRNA in tumors representative of IHA-inconsistent* (lanes 1 *to* 5) *and IHA-consistent* (lanes 6 *to* 10) *tumor subgroups. RNA extracted from tumors was analyzed by triple-primer PCR as described above.* Upper *and* lower arrows *show wild-type and clone 4 corresponding signals, respectively.*

## Discussion

Using PCR-based approaches that allow the investigation of the prevalence of different exon-deleted and truncated ER variant mRNAs within breast tumor samples, we have investigated ER variant mRNA expression within 63 breast tumors that presented similar (IHA-consistent) or different (IHA-inconsistent) results when assessed for ER expression by IHA performed with an antibody (1D5) recognizing the amino terminal as compared with an antibody (AER311) targeting the carboxy terminal of the ER protein. We have found that, whereas variants such as D7-ER, D4-ER, D3–4/ER, and D4/7-ER are detected at the same frequency in IHA-inconsistent and IHA-consistent breast tumors, other variants, including D2–3/7-deleted, D2–3-4-ER, and D-3–7-ER, are preferentially detected in IHA-inconsistent cases. This difference between subgroups was statistically significant for two of these variants: exon-2–3-4-deleted ER and exon-3–7-deleted ER. Both of these two variant mRNAs possess sequence modifications that introduce a shift in the WT-ER coding sequence that would encode ER-like proteins containing the amino-terminal TAF-1 transactivation domain but missing all the carboxyl-terminal extremity of WT-ER protein (Figure 1). These putative variant ER proteins would therefore theoretically be recognized by 1D5 antibody but not AER311 antibody. Furthermore, detectable expression of the subset of variant mRNAs able to encode truncated ER-like proteins (except the uniformly prevalent D7-ER variant that was detected in all but 6 tumors of the 63 studied) was significantly higher in the IHA-inconsistent tumor group. In contrast, detectable expression of variants encoding in-frame proteins that should be recognized by both antibodies was no different between tumor subgroups. Taken together, these results are in keeping with the hypothesis that ER variant mRNAs encoding truncated ER proteins may participate in the synthesis of ER-like proteins differentially recognized by 1D5 and AER311 antibodies. This assumption is also further supported by the results obtained using a quantitative PCR-based approach applied to the same tumors, which indicate that IHA-inconsistent tumors also possess significantly higher levels of ER clone 4 truncated variant relative to WT-ER compared with IHA-consistent tumors.

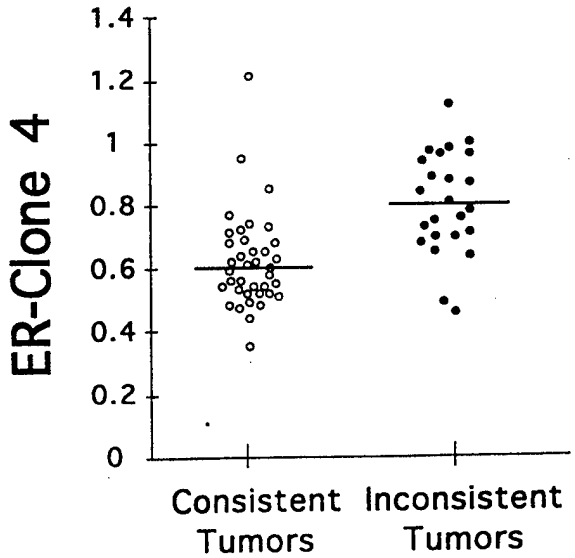


Figure 5. *Comparison of the relative expression of ER clone 4 variant mRNA in IHA-inconsistent breast tumors and in IHA-consistent breast tumors. For each sample, the mean of three independent measures of clone 4 expression was expressed as a percentage of the corresponding wild-type ER signal. The difference between two groups is statistically significant ($P < 0.01$, Mann Whitney rank sum test, two sided).*

Until the development of antibodies that are specific for individual ER variant proteins, the premise that proteins encoded by ER variant mRNAs may directly interfere with ER immunodetection and determination of ER status by IHA remains to be proven. It is clear from *in vitro* laboratory studies that ER variants can encode proteins that possess a variety of dominant negative, positive, or undetectable activities when tested for their ability to interfere with transactivation of classical ER enhancer sequences/elements.[6,9,19] Thus, although we and others[4,5] have observed a relative increase in amino-terminal signal that may correspond to increased truncated ER proteins, the functional implications in terms of response to endocrine therapy will depend

on the nature of the specific ER variant activities in a given tumor.

Although a good correlation between ER-DCC and ER-IHA is often found, approximately 20% of cases are discordant.[5] It is believed that the cause of this discordance is multifactorial and both ER-DCC-positive/IHA-negative and ER-DCC-negative/IHA-positive cases have been attributed overall to tumor heterogeneity, sampling, variable frozen tissue handling, and formalin fixation.[20] However, an explanation for discordant results is not always apparent in specific cases.[20] Thus, although recent studies have shown that immunodetection using 1D5-IHA can accurately predict endocrine response of breast cancer,[21] the relative predictive value of ER-DCC *versus* ER-IHA is still under debate.[20–23] In the light of our results, and laboratory evidence to suggest that ER variant proteins encoded by ER variant mRNAs may participate in endocrine response,[6–14] it may be important to assess ER variant expression in future studies concerning ER-IHA status and response to endocrine therapy.

Interestingly, the two exon-deleted ER variant mRNAs, the expression of which was shown here to be correlated to inconsistent results by IHA (ie, D2–3-4-ER and D-3–7-ER), have not been detected until recently.[15] However, our previous studies[5] indicate that expression of these variants may be associated with high-grade tumors and high ER level, respectively.[15] Similarly, we have shown that a higher level of ER clone 4 mRNA expression correlates with tumor progression and poor prognosis.[16,24] This suggests not only that these ER variant mRNAs may contribute to discrepant IHA results but also that alteration of their expression is associated with tumor progression.

In conclusion, we have found a significant correlation between expression of certain ER variant mRNAs and inconsistent IHA results after assessment and comparison of ER expression with antibodies directed to either amino- or carboxyl-terminal epitopes in human breast cancer. These data add to the growing body of evidence that suggests that ER variants may be translated *in vivo* into ER-like proteins.[5,25,26] Finally, these results suggest that ER variant expression may be an important parameter to consider in the determination of ER status in human breast cancer.

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# APPENDIX 9

# Oestrogen Receptor Variants and Mutations in Human Breast Cancer

Leigh C. Murphy, Etienne Leygue, Helmut Dotzlaw, Deborah Douglas, Amanda Coutts and Peter H. Watson[1]

**Several oestrogen receptor variant and mutated mRNA species have been identified in human breast samples and cell lines. Over-expression and altered expression of some of these mRNAs have been correlated with breast tumourigenesis and progression. The following review focuses on the current knowledge available in the scientific literature with respect to the type and characteristics of oestrogen receptor variants and mutations that have been identified as occurring naturally in human breast tissues and cell lines.**

**Key words: breast cancer; mutations; oestrogen receptor.**

**(Annals of Medicine 29: 221–234, 1997)**

## Introduction

Oestrogens are major regulators of mammary gland development. However, oestrogens are also involved in the growth and progression of mammary cancers (1). The principal mechanism by which the effects of oestrogen are mediated in either normal or neoplastic target cells is via an initial interaction with the oestrogen receptor (ER). This protein is an intracellular ligand-activated transcription factor regulating the expression of several gene products, which ultimately result in target-tissue-specific oestrogen responses. The ER can be divided into several domains, labelled A–F, starting from the N-terminus (2, 3). Mutational analyses have defined several functional regions within each domain. The A/B region contains a cell- and promoter-specific, ligand-independent nonacidic transactivating function (AF-1), which may have a role in the agonist activity of the tamoxifen-like antioestrogens (4–6). The C domain contains two zinc finger motifs, which are responsible for the specific DNA-binding activity of the protein (2, 3). The C domain also contains an apparently constitutive dimerization domain (2). The D domain is thought to be a flexible hinge region but also contains a number of basic amino acids conserved in all receptors, which may have a role in nuclear localization and DNA binding (2). The E domain contains the ligand-binding domain, a ligand-dependent dimerization activity and a ligand-dependent nonacidic transactivating function (AF-2). The carboxy terminal F domain was originally thought to have no functional significance; however, more recent analyses suggest that it has a specific modulatory function on transcriptional responses to oestrogens and antioestrogens that is influenced by cell context (7). Upon oestrogen binding the receptor undergoes conformational changes resulting in its 'activation', so that it forms stable homodimers that bind tightly to specific nucleotide sequences called oestrogen-responsive elements, or EREs (2, 3). EREs are usually found in the promoter region of those genes the transcription of which is regulated by oestrogen. In this way oestrogen can alter the transcription of several genes that ultimately lead to DNA synthesis and proliferation of breast cancer cells.

However, the involvement of oestrogen in mammary tumour growth and progression is thought to involve, at some stages, perturbations of the ER signal transduction pathway, which are likely to contribute to tumour progression and the eventual development of hormone independence and a more aggressive phenotype (8–10). One mechanism underlying such perturbations could be alterations in the structure and therefore function of the ER itself. This review will focus on structural changes in the ER that have been identified as occurring naturally in human breast tissues and cell lines.

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## Identification and Structure of ER Variant and Mutant mRNA Species

Molecular evidence for the potential existence of variant and/or mutant ER proteins has been obtained by analysis of ER-like mRNA in normal and neoplastic breast tissues. Many different types of ER-like mRNAs distinct from the wild-type ER mRNA have now been identified in several tissues and cell lines, including both normal and neoplastic human breast cells and tissues. It should be remembered, however, that few of these ER-like transcripts have been cloned and characterized from cDNAs representing full-length transcripts. Indeed little, if any, attention has been paid to the presence or absence of a 3′-untranslated region, a polyadenylation signal and a poly A tail. Given these caveats to interpretation, several different patterns of ER-like mRNA have been found or predicted, as described in the following.

### *Transcripts Containing Precise Single or Multiple Exon Deletions*

Multiple ER-like transcripts have been identified that contain precise exon deletions (11–20). Several of the exon-deleted transcripts that have been described in the literature are shown in Figure 1. The majority of these have been identified by reverse transcription–polymerase chain reaction (RT–PCR) approaches, which by virtue of specific primer design have focused on small regions of the known wild-type ER mRNA. More recently, however, ER-like transcripts containing two or three entire exon deletions have been detected in cell lines and tissue samples (16–20) and amongst the deletion-type ER variants, this type of variant ER transcript appears now to be the most predominant. However, the identification of multiple types of exon deleted transcripts in any one cell line or tissue sample (16–18, 20) underscores the need to study these variant ER transcripts altogether, as well as individually.

### *Other Deleted Transcripts*

ER-like transcripts containing variable-sized deletions that are not entire exon deletions have also been detected. This type of alteration falls into two groups: one in which a single nucleotide has been deleted (21, 22), and the other in which several hundreds of continuous nucleotides have been deleted but starting and ending within known exon sequences (Fig. 1, Table 1) (16, 20, 21, 23).

### *Truncated Transcripts*

These altered ER-like transcripts are significantly smaller than the wild-type ER mRNA as determined by Northern blot analysis (24). cDNA cloning of apparently full-length or near to full-length transcripts was used to characterize these transcripts fully. These transcripts contain entire exon sequences of at least 2 of the 5′ ER exon sequences, and then diverge into ER-unrelated sequences (25), some of which appear to be LINE-1

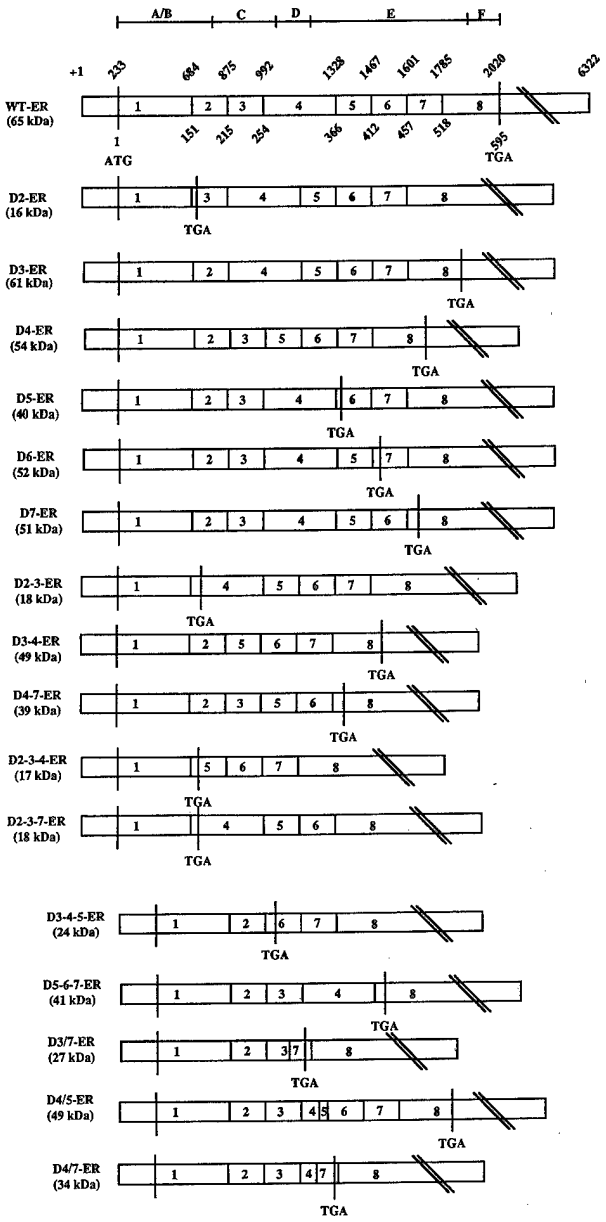


**Figure 1.** Schematic diagram of the wild-type human oestrogen receptor (WT-ER) cDNA, which contains eight different exons coding for a protein divided into structural and functional domains (A–F). Region A/B of the receptor is implicated in transactivating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another transactivating function (AF-2). The numbering on the top of the cDNA refers to the nucleotide position as defined in (64). Below the WT-ER cDNA are the various putative exon and other large deleted ER cDNAs. ATG shows the translation initiation codons, TGA shows the inframe translation termination codons and the numbering below the cDNA refers to the amino acid positions as defined in (64). D indicates deletion, and the estimated molecular mass (kDa = kiloDaltons) of each open reading frame is shown in parentheses. Molecular masses were estimated using MacVector version 4.1.4 software.

**Table 1. ER variants identified in human breast tissues and cell lines.**

| Variant mRNA | Estimated $M_r$ of predicted protein (kDa) | Functional domains | Reference |
|---|---|---|---|
| Wild-type ER | 65 | A, B, C, D, E, F | (4, 5, 64) |
| D2-ER | 16 | A, B? | (11, 16–19) |
| D3-ER | 61 | A, B, D, E, F | (11, 14) |
| D4-ER | 54 | A, B, E?, F | (12, 17–20, 59) |
| D5-ER | 40 | A, B, C, D | (13) |
| D6-ER | 52 | A, B, C, D | (14) |
| D7-ER | 51 | A, B, C, D | (11, 15, 20) |
| D2-3-ER | 18 | A, B? | (16, 20) |
| D3-4-ER | 49 | A, B, E?, F | (16, 17, 20) |
| D4-7-ER | 39 | A, B | (18–20) |
| D2-3-4-ER | 17 | A, B? | (20) |
| D2-3-7-ER | 18 | A, B? | (20) |
| D3-4-5-ER | 24 | A, B | (18) |
| D5-6-7-ER | 41 | A, B, C | (18) |
| D3/7-ER | 27 | A, B | (20) |
| D4/5-ER | 49 | A, B, C?, F | (21) |
| D4/7-ER | 34 | A, B, C? | (23) |
| Clone 4-ER | 24 | A, B | (25) |
| Clone 24-ER | 37 | A, B | (25) |
| Exon $6^2$-ER | 51 | A, B, C, D | (26) |
| Exon $(34)^2$-ER | 75 | A, B, C+, D+, E, F | (26) |
| Exon $(67)^2$-ER | 80 | A, B, C, D, E+, F | (27, 45) |
| ER-69-bp | 69 | A, B, C, D, E?, F | (26, 28) |

? Indicates that an alteration of the function has been shown or is likely to occur.

related (Fig. 2, Table 1). Although several different truncated ER mRNAs have been cloned, some of these were only found to be expressed in a single breast tumour, although others, such as the clone 4-truncated ER mRNA, have been found to be expressed in many human breast tumours (25).

### *Insertions*

ER-like transcripts have been identified containing variable-sized nucleotide insertions. Such insertions consist of one to two nucleotides (21, 22), larger insertions of 69 and more nucleotides (21, 26), and apparently complete exon duplications (26, 27) (Fig. 2, Table 1). These abnormal ER-like transcripts were detected using RT–PCR analyses, and further studies showed that the exon 6 plus 7-duplicated ER-like transcript was generated from a mutated ER gene in which genomic rearrangement resulted in the duplication of exons 6 and 7 in an in-frame fashion (27). As well the 69-bp-inserted ER mRNA is probably generated from a point mutation in one allele of the ER gene in the breast tumour from which it was cloned. This point mutation generates a consensus splice donor site at the 3′ end of the 69-bp sequence present in intron 5. In addition, a splice acceptor consensus sequence is normally present at the 5′ end of the 69-bp sequence, and thus the 69 sequences are likely to be seen as another exon in the gene (28).

### *Point Mutations*

Several point mutations including silent polymorphisms have been identified in ER-like transcripts (Table 2) (21,

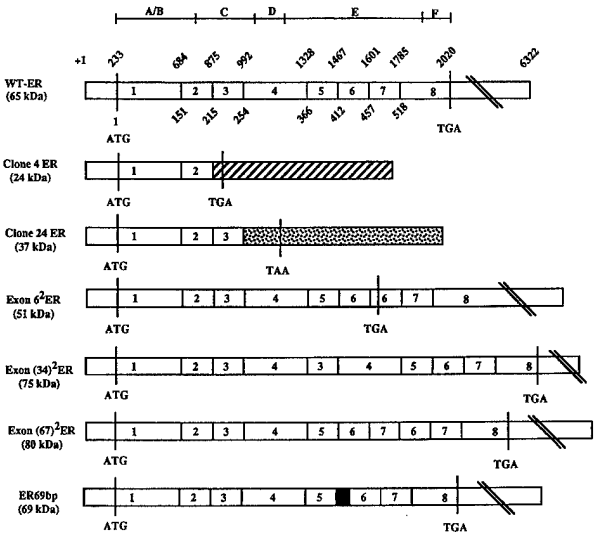


**Figure 2.** Schematic diagram of the wild-type human oestrogen receptor (WT-ER) cDNA, which contains eight different exons coding for a protein divided into structural and functional domains (A–F), as described in Figure 1. The numbering on the top of the cDNA refers to the nucleotide position as defined in (64). Below the WT-ER cDNA are the clone 4- and clone 24-truncated ER cDNAs, which have been cloned previously (25), as well as the putative cDNAs representing exon-duplicated and some inserted ER mRNAs (26, 27). ATG shows the translation initiation codons, TGA shows the in-frame translation termination codons and the numbering below the cDNA refers to the amino acid positions as defined in (64). The estimated molecular mass (kDa = kiloDaltons) of each open reading frame is shown in brackets. Molecular masses were estimated using MacVector version 4.1.4 software.

**Table 2. Small insertions/deletions and point mutations/polymorphisms identified in the oestrogen receptor mRNA.**

| Nucleotide change | Exon | Amino acid change | Functional domains | Reference |
|---|---|---|---|---|
| 262 T→C | 1 | 10 Ser no change | A, B, C, D, E, F | (25, 29) |
| 439 C→G | 1 | 69 Asn→Lys | A, B?, C, D, E, F | (29) |
| 493 G→C | 1 | 87 Ala no change (B variant) | A, B, C, D, E, F | (29–32) |
| 701 C→T | 2 | +Stop after 156 | A, B? | (36) |
| 961 C→T | 3 | 243 Arg no change | A, B, C, D, E, F | (29) |
| TT insert after 981 | 3 | Met 250→Ile+stop | A, B | (21) |
| 1059 C→T | 4 | 276 Gly no change | A, B, C, D, E, F | (22) |
| 1119 T→C | 4 | 296 Leu→Pro | A, B, C, D?, E?, F | (32) |
| 1207 C→G | 4 | 325 Pro no change | A, B, C, D, E, F | (29, 32) |
| 1283 G→T | 4 | 352 Asp→Tyr | A, B, C, D, E?, F | (34, 35) |
| 1290 A→T | 4 | 353 Glu→Val | A, B, C, D?, E?, F | (22) |
| 1418 A→G | 5 | 396 Met→Val | A, B, C, D, E?, F | (29) |
| 1463 G del | 5 | 411 Asp→Thr+6 extra novel a.a. | A, B, C, D | (21) |
| del T at 1526 | 5 | 432 Ser→His+4 extra novel a.a. | A, B, C, D | (22) |
| 1503–1550 replaced | 6 | 424 Ile→Arg+28 | A, B, C, D | (22) |
| by 1380–1422 | 5 | extra novel a.a. | | |
| 1647 G→A | 7 | 472 Lys no change | A, B, C, D, E, F | (22) |
| 1747 C→G | 7 | 505 Ala no change | A, B, C, D, E, F | (22) |
| 1963 T→C | 8 | 577 His no change | A, B, C, D, E, F | (22) |
| 2014 A→G | 8 | 594 Thr no change | A, B, C, D, E, F | (29) |

Nucleotides are numbered according to the start site of transcription (+1) in (64). ? Indicates that an alteration of the function has been shown or is likely to occur.

22, 29–35). The only known germline mutation in the human ER associated with disease is a point mutation (36) identified in a young adult male presenting with osteoporosis, unfused epiphyses, continued linear growth in adulthood, and oestrogen resistance. Furthermore, only approximately 1% of primary breast tumours have point mutations in the ER gene (22, 29), which in some cases might be linked to hereditary breast cancer (37).

The above ER-like mRNA molecules have, in most cases, been identified in human breast cancer tissues or human breast cancer cell lines. However, data are now emerging showing that several of the exon-deleted and truncated transcripts are also expressed in multiple samples of normal human breast tissue (16–18). This suggests that the mechanisms for generating these transcripts are present in normal human mammary cells and therefore these transcripts are normal variants, and probably generated by an alternative splicing mechanism (38). It is less likely that the inserted transcripts and many of the amino acid altering point mutations are normal variants. There is a greater likelihood that such transcripts were generated from a mutated ER allele present in some human breast tumours (27, 28). In summary, a large body of molecular data exists to support at least the potential for the existence of variant or abnormal ER-like proteins in human breast cancer.

## Expression of Multiple ER Variant mRNAs in Human Breast Tissues

The identification of several ER variant mRNAs in normal human breast tissues implies that either the variant mRNAs or their respective proteins may have a normal role in ER signal transduction. Consequently, changes in the balance of ER-like molecules could perturb the ER signalling pathway and contribute to tumour progression. It has therefore become important to determine whether levels and the pattern of ER variant expression are different between normal and neoplastic breast tissues, as well as amongst groups of tumours with different characteristics.

This has been studied initially by investigating individual variant ER mRNA levels relative to wild-type ER mRNA levels. The relative expression of the truncated clone 4 ER mRNA (39) and the exon 5-deleted ER mRNA, but not the exon 7-deleted ER mRNA (16) were found to be significantly elevated in breast tumour tissue compared with normal breast tissue. It has also been suggested that the level of the exon 3-deleted ER mRNA is reduced in breast tumour tissue compared with normal tissue (40). Such data suggest that the expression of some but not all variant ER mRNAs is deregulated during breast tumourigenesis.

Investigation of the relative expression of the truncated clone 4 ER variant in groups of breast tumours with different prognostic characteristics (41) identified a statistically significant increased expression of this transcript in breast tumours with combined characteristics of poor prognosis (node positive, large tumour size, high S-phase fraction) and lack of endocrine sensitivity (progesterone receptor (PR) negative). Elevated exon 5-deleted ER transcripts have been found in ER−/PR+ and ER−/pS2+ tumours (42), while increased levels of the exon 7-deleted ER mRNA are often found in ER+/PR− breast tumours (15).

These data suggest that altered expression of some ER variants is associated with different phenotypes in

human breast tumours and may have a functional role in such phenotypes. However, it has become increasingly apparent that several ER variant mRNAs can be detected in any one sample of either normal or cancerous breast tissues (16–18). While it is unclear whether any or all of these mRNAs are stably translated *in vivo* (see discussion below), many of the predicted ER-like proteins are lacking some functional domains (4) of the wild-type ER (Figs 1 and 2), and some have been shown to exhibit altered functions *ex vivo*. Therefore, the possibility exists that several ER variant proteins could be expressed together (16–18) and the validity of investigating individual variants in isolation can be questioned. Furthermore, previous analyses have depended largely on assays that focus on limited regions of the transcript, and would be unlikely to detect more than one modification per individual variant mRNA. However, it is now clear that more than one modification can occur in variant transcripts (19). Thus signals attributed to the exon 7-deleted ER variant mRNA, detected by RT–PCR using primers in exon 5 and 8 or by RNAse protection assays with probes covering the exon 6/8 junction, may also include contributions from a variant deleted in both exon 4 and 7, recently identified by Madsen et al. (19). Nevertheless, these molecules may result in quite different proteins that differ in activity and may modulate differentially the ER signalling pathway. There is thus a need to investigate qualitatively and quantitatively the expression of total ER variant mRNAs within a single tumour. An attempt to address this issue was published recently (20). A strategy was developed to allow the investigation of known and unknown exon-deleted or inserted ER variant mRNAs in any one tissue sample as well as to determine possible changes in the relative expression of such variants amongst themselves and with respect to the wild-type ER transcript. The approach (20) used is illustrated in Figure 3; however, owing to practical limitations it cannot measure all types of ER variants, and indeed the truncated transcripts would not be included in such an analysis (25, 39). A competitive amplification occurs amongst all exon-deleted or inserted ER variant transcripts, which depends on their initial relative expression, and the detection of bands corresponding to specific ER variants reflects the relative expression of these ER variant mRNA species within the samples. A survey of 100 breast tumours (20), showed that the most frequently expressed ER variants at a relatively high abundance were the exon 7-deleted variant, the exon 4-deleted variant, a variant deleted in both exons 3 and 4, a variant deleted in exons 2, 3 and 7, a variant deleted in both exons 4 and 7, a variant deleted in exons 2, 3 and 4, and a variant deleted from within exon 3 to within exon 7. Neither the exon 5-deleted nor the exon 3-deleted ER mRNAs were detected using this approach. Interestingly, preferential detection of some deleted variants was found to be associated with known prognostic markers in breast cancer (20).

In summary, data exist to support the hypothesis that altered expression of variant ER mRNA expression occurs during both breast tumourigenesis and breast cancer progression.

## Expression of Variant or Mutant ER Proteins

It is unclear at this stage whether all or any of the ER-like transcripts so far identified are stably translated *in vivo*. It is certainly possible for many of them to be expressed at high levels from expression constructs transfected into mammalian, yeast and bacterial host cells. Furthermore, in some cases ER variant expression under these conditions has identified a putative function of the resulting variant protein (11, 13, 15). For example, exon 3 and exon 7-deleted variants may act as dominant negative regulators (inhibitors) of wild-type ER (11, 40) whereas exon 5-deleted ER has ligand-independent transcriptional activity (13, 43) (see discussion below).

More importantly, an ER-like protein consistent with that predicted to be encoded by the exon 5-deleted ER transcript has been found to be expressed naturally in some BT-20 human breast cancer cell lines (44). In addition, an immunoreactive 80 kDa ER-like protein has

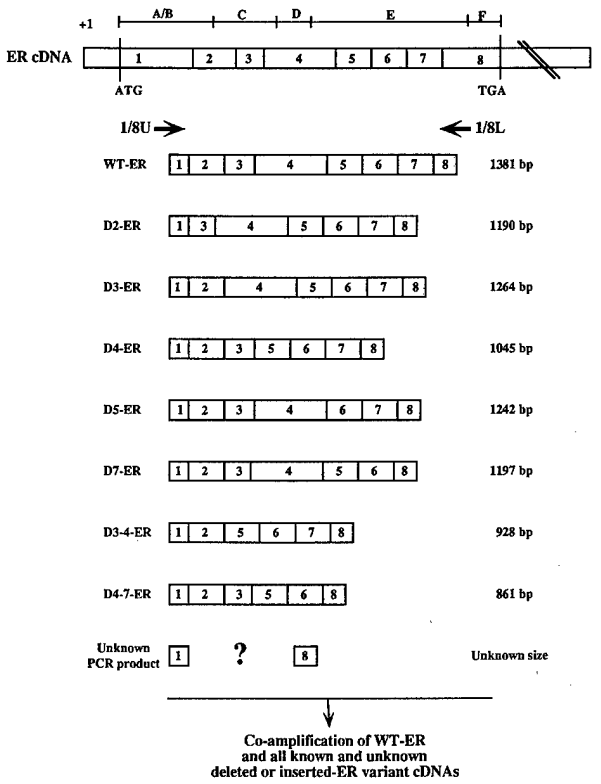


**Figure 3.** Schematic representation of wild-type oestrogen receptor (WT-ER) cDNA and primers allowing coamplification of most exon-deleted ER variants. 1/8U and 1/8L primers allow amplification of a 1381 bp fragment corresponding to WT-ER mRNA. Coamplification of all possible exon-deleted or inserted variants that contain exon 1 and 8 sequences can occur (20). Amplification of the previously described ER variant mRNAs deleted in exon 2 (D2-ER), exon 3 (D3-ER), exon 4 (D4-ER), exon 5 (D5-ER), exon 7 (D7-ER), both exons 3 and 4 (D3-4-ER), exons 2 and 3 (D2-3-ER) and exons 4 and exon 7 (D4/7-ER) would generate 1190 bp, 1264 bp, 1045 bp, 1242 bp, 1197 bp, 928 bp, 1073 bp and 861 bp fragments, respectively.

been identified in an MCF-7 subclone (45). This protein corresponds to the predicted protein encoded by an ER-like transcript containing an exon 6 and 7 duplication, which was cloned from the same cell line (27). These data demonstrate the ability of some ER-like transcripts to be naturally translated into stable proteins, which can be detected by current methods, and suggest the likelihood of other ER-like transcripts being stably translated *in vivo* under natural conditions.

Other studies support the expression of variant or mutant ER-like proteins but their relationship to known variant or mutant ER mRNA remains unclear. Immunohistochemical staining with a polyclonal antibody was used previously to identify two types of apparently defective ER in human breast cancers (46); one that bound the nucleus in a ligand-independent fashion and one that could not bind to the nucleus even in the presence of ligand. Several other studies have identified ligand-binding forms of the ER that have both altered molecular mass (often truncated compared to the wild-type ER) and altered isoelectric points (47). The correlation of some of these ER-like proteins with biological parameters suggests that they may play a role in the ER signal transduction pathway (48). More recently, truncated DNA-binding forms of ER-like proteins have been identified in some human breast cancer biopsy samples (49). ER antibodies (Fig. 4) recognizing epitopes in the A/B and E domains of the wild-type receptor were found to detect these truncated ER-like proteins. An ER-like protein was identified in some ER+/PR− human breast tumours that formed complexes with an oligonucleotide containing an ERE in gel shift assays (15). The complex was supershifted by H226 and H222 antibodies but not by the D75 antibody recognizing a more C-terminal epitope (Fig. 4). Steroid hormone-induced mammary tumours in Grunder mice progress from hormone dependence to hormone independence following serial transplantation. This progression is associated with decreased expression of the 65 kDa ER protein and a marked increase in tamoxifen aziridine-bound, immunopurified 50 and 35 kDa proteins (50).

The relationship of any of these ER-like proteins that have been characterized in some human and mouse tumour tissues to ER-like proteins that are potentially encoded by some of the previously characterized ER-like mRNAs is unclear and remains to be elucidated. Very few, if any, Western blotting analyses using differential antibody detection of ER-like proteins in human breast tumours have been reported. One study, where ER antibodies recognizing epitopes within the ligand-binding domain were used for Western blotting, identified larger than wild-type as well as smaller than wild-type ER immunoreactive proteins (49, 51). However, many of the known variant ER transcripts are likely to encode proteins around the size of heavy and light immunoglobulin chains. Immunoglobulin contamination of human breast tumours and immunoprecipitated complexes would probably interfere with Western blot analysis of such variant ER proteins (51).

More recently, a group of human breast tumours were analyzed immunohistochemically (52) for ER expression by using antibodies that recognize either an N-terminally localized epitope in the wild-type ER protein, or a C-terminally localized epitope in the wild-type ER protein (Fig. 4). It was found that the antibody recognizing the C-terminally localized epitope correlated better with the ligand-binding assays performed on adjacent tissues than did the antibody recognizing the N-terminally localized epitope. Additionally, although in many tumours the immunohistochemical results using each antibody showed good concordance, in some tumours the results were discordant, with the signal tending to be higher with the N-terminal antibody (53). Because many of the proteins predicted from variant ER mRNAs would be truncated at the C-terminus and would not contain the epitope recognized by the C-terminal antibody, one interpretation of these data would be that truncated variant ER proteins are more highly expressed in the discordant group of tumours. This hypothesis was tested by investigating the pattern and relative expression of variant ER mRNAs in the discordant and concordant groups of breast tumours. Several ER variant mRNAs that encode putative short ER-like proteins that would be recognized only by an N-terminal-targeted antibody were preferentially and more highly expressed in the discordant breast tumour group. These ER variants were: the clone 4-truncated ER mRNA; the exon 2, 3 plus 7-deleted ER mRNA; the exon 2, 3 plus 4-deleted ER mRNA; and the variant deleted within exon 3 to within exon 7 (53). The data suggest that the ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER measured by immunodetection assays using N- or C-terminal antibodies. Further, the data are consistent with the ability of ER variant mRNAs to be stably translated *in vivo* and therefore have a functional role or roles in ER signal transduction.

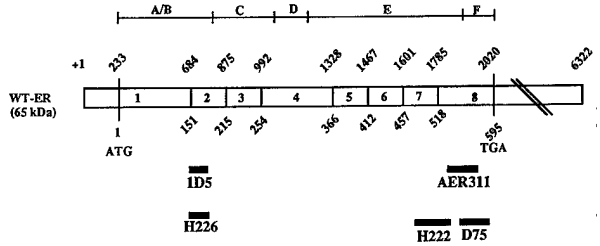


**Figure 4.** Approximate location of the epitopes recognized by the various oestrogen receptor antibodies (1D5, H226, H222, AER311, D75) referred to in this review. Schematic diagram of the wild-type human oestrogen receptor (WT-ER) cDNA, which contains eight different exons coding for a protein divided into structural and functional domains (A–F). The numbering on the top of the cDNA refers to the nucleotide position as defined in (64) and the numbering below the cDNA refers to the amino acid positions as defined in (64).

## Structure and Function *In Vitro* and *In Vivo* of Putative Variant and Mutant ER-like Proteins

The data summarized in the previous section suggest the likelihood of some or all ER-like transcripts, being

stably translated *in vivo*. This provides a rationale for discussing the possible structure and function of the proteins predicted to be expressed from variant and/or mutant ER-like mRNAs.

### 1. Exon 7-deleted ER

Relative to all other deleted ER transcripts the exon 7-deleted ER variant appears to be the most abundant in human breast tissues (20). This transcript was first identified in T-47D human breast cancer cells (11) and was subsequently found in human breast tumour samples (15). The predicted protein encoded by this variant ER transcript is approximately 51 kDa (Fig. 1, Table 1), and is identical to the wild-type ER protein up to amino acid residue 456 and thereafter encodes 10 novel amino acids. The putative protein encoded by the exon 7-deleted transcript would therefore be truncated in the E domain, which includes the ligand binding, AF-2 and a strong dimerization domain of the wild-type receptor. The putative function of the protein encoded by the exon 7-deleted transcript is controversial. Wang and Miksicek (11) using HeLa cells found that it did not bind ERE DNA or have transcriptional activity of its own. Moreover, under these conditions the exon 7-deleted ER did not affect the activity of the wild-type ER. These data contrast with those obtained by Fuqua et al. (15), where, using a yeast expression system, the exon 7-deleted ER protein was found to inhibit wild-type ER activity. Furthermore, Fuqua et al. had originally isolated the exon 7-deleted ER variant mRNA from an ER+/PgR− breast tumour that contained an ER-like protein able to bind to DNA containing an ERE as determined by gel mobility shift analysis, but that interacted differentially with ER antibodies suggestive of an ER-like protein with a C-terminal truncation (15). The data of Fuqua et al. were consistent with the idea that overexpression of an exon 7-deleted ER protein could contribute functionally to the ER+/PR− breast tumour phenotype. The hypothesis was further supported by the observation that exon 7-deleted mRNA levels were significantly elevated in a group of human breast tumours that were ER+/PgR−/pS2− compared to those which were ER+/PgR+. Although exon 7-deleted mRNA was found in normal breast tissue, its expression was not significantly different in normal versus breast tumour tissue, although in the same tissue samples the levels of both the exon 5-deleted and the clone 4-truncated ER mRNAs were significantly higher in tumours compared to normal breast tissues (16). This latter study, in contrast to studies reported by Fuqua et al., did not find any significant relationship between exon 7-deleted ER mRNA level and PR status or tumour grade (16, 20).

The reported data suggest that the activity of the exon 7-deleted ER may vary in a cell-type and promoter-specific fashion. This in turn suggests that the background milieu may dictate the impact of variant ERs. So, although there are a few reports of altered exon 7-deleted ER expression, it is one of the most abundantly expressed variants in human breast tissues and activity of this variant may depend on an altered cellular milieu.

### 2. Exon 5-deleted ER

The predicted protein from the exon 5-deleted ER mRNA is a truncated protein of approximately 50 kDa as a stop codon has been introduced after amino acid residue 371 (Fig. 1, Table 1). Its amino acid composition would be identical to the wild-type ER up to amino acid 366 followed by five novel amino acids, and consequently the majority of the ligand-binding domain of the wild-type ER will be missing.

Using a yeast expression system Fuqua et al. (13) showed that the exon 5-deleted ER displayed ligand-independent transcription from an ER-regulated reporter plasmid. Rea and Parker (54) confirmed this result in chicken embryo fibroblasts. However, when an exon 5-deleted ER expression vector was stably transfected into MCF-7 cells, it had no effect on an ERE-tk-luciferase reporter plasmid, it slightly increased transcription from an $ERE_2$TATA-CAT but not an $ERE_1$TATA-CAT reporter plasmid, and it had no effect on endogenous oestrogen-responsive genes such as pS2 and progesterone receptor. Neither did it result in the development of oestrogen independence and antioestrogen resistance in these cells. In contrast, a similar study by Fuqua and Wolf (55) showed that over-expression of the exon 5-deleted ER protein resulted in increased progesterone receptor levels in the absence of oestrogen, as well as oestrogen-independent growth and tamoxifen resistance. The reasons for the different results between the two groups are unclear, although differences in the original parent MCF-7 cells was suggested, in turn suggesting that other changes in addition to altered exon 5-deleted ER expression are required for hormonal progression in human breast cancer cells. This is not unreasonable as several mechanisms, either alone or in combination, may be responsible for such progression (10). For example, it is possible that the alteration of growth factors or their cognate receptors, some of which have been shown to result in ligand-independent activation of the wild-type ER through the N-terminal AF-1 domain (56, 57), may also be required in conjunction with altered expression of ER variants. Interestingly, Klotz et al. (58) identified a correlation between increased expression of the exon 5-deleted ER transcript relative to the wild-type ER and reduced responsiveness to oestrogen in MCF-7 stocks obtained from various laboratories in North America.

Measurement of the exon 5-deleted ER mRNA in clinical samples provides further insight into a possible role for this ER variant. The exon 5-deleted ER transcript was found to be present in normal human mammary tissue, but its level relative to the wild-type ER mRNA was significantly increased in breast tumour tissues (16). The exon 5-deleted ER transcript was first identified in a tumour that was ER−/PgR+, a finding consistent with the speculation concerning the ligand-independent activity of a protein encoded by this transcript (13). Using a specific RT–PCR approach, this transcript has been found to be more highly expressed,

and in some cases more abundant, than the wild-type ER mRNA, in ER−/PgR+ breast tumours (13, 42). However, when measured within a wide range of ER+/PR+ breast tumours, using a long-range RT–PCR approach (20), its relative expression with respect to all other deleted transcripts is low to undetectable (20, 59). Again using a specific RT–PCR analysis, Daffada et al. (42) found significantly higher levels of the exon 5-deleted ER transcript in those human breast tumours that were ER−/PgR+ or ER−/pS2+. However, while levels of the exon 5-deleted ER transcript are found to vary widely in human breast tumours, no significant differences in their levels were found between tamoxifen-resistant and tamoxifen-sensitive tumours (42). Furthermore, in a tamoxifen-resistant MCF-7 cell line the level of the exon 5-deleted transcripts was lower than the sensitive parent line (19) although differential expression of other ER variants was found. Presently no clear-cut correlation between exon 5-deleted ER expression and tamoxifen resistance is evident. However, this might be expected because there are multiple variants expressed in any one tumour and multiple mechanisms are likely to be involved in the development of tamoxifen resistance in particular and endocrine therapy resistance in general (10).

In conclusion, the putative activity of the protein encoded by the exon 5-deleted ER mRNA could contribute to the development of oestrogen independence and endocrine resistance in human breast cancer. Certainly correlations between the level of this transcript and apparently constitutively elevated oestrogen target gene expression would support this hypothesis. However, differences between the phenotypes generated by stable transfection studies and the lack of correlation of this transcript with tamoxifen-resistant breast tumours suggest that other factors are probably involved, either together with or independently of elevated exon 5-deleted ER expression. Although the naturally occurring exon 5-deleted ER mRNA and its putative cognate protein have been the most widely studied ER variants to date, we now know that multiple ER variants can be found in both normal and neoplastic breast tissues (16–18). Furthermore, using assays that allow the investigation of the relative expression of multiple ER variant mRNAs, it is apparent that the exon 5-deleted transcript represents one of the lower abundance variant transcripts in a wide range of human breast tumours, except perhaps in the ER−/PR+ phenotype.

### 3. *Exon 4-deleted ER*

The exon 4-deleted ER transcript has been found expressed in human breast cancer cell lines (12, 19, 59), human breast cancer tissue (17, 18, 20) and normal human breast (17, 18). This transcript contains an in-frame deletion and is predicted to encode a protein of approximately 54 kDa (Fig. 1, Table 1) which would be missing a strong nuclear localization domain and a portion of the E domain of the wild-type ER. When an expression vector was made for this variant, the encoded protein did not bind oestradiol or an ERE, and had no transcriptional activity of its own nor any dominant negative activity against the wild-type ER (61, 62). Although these studies suggest that an exon 4-deleted ER is essentially inactive, negative results may reflect the promoter and cell types used in these studies. More recently, a correlation was found between the relatively increased expression of the exon 4-deleted ER mRNA with high PR expression and low grade, suggesting its correlation with some good prognostic features in human breast tumours (20). However, any functional role that this ER variant might have in this correlation is as yet unclear.

### 4. *Exon 3-deleted ER*

An exon 3-deleted ER transcript was initially identified in T-47D human breast cancer cells (11). A deletion of exon 3 from the wild-type ER transcript is in frame and generates a protein of approximately 61 kDa that lacks the second zinc finger of the wild-type ER DNA-binding motif. The function of the putative protein encoded by this transcript is controversial. When expression vectors for this protein were transfected into HeLa cells the exon 3-deleted protein demonstrated a dominant negative activity, inhibiting wild-type ER transcriptional activity, without any intrinsic transcriptional activity of its own. This variant ER, while unable to bind to an ERE in a gel mobility shift assay, inhibited the ability of the wild-type ER to bind to an ERE under the same conditions (11, 14). Preliminary data in which this variant was stably over-expressed in MCF-7 human breast cancer cells suggest that it has dominant negative activity in this model as well. Over-expressing cells were growth-inhibited by oestrogen, suggesting that this variant can inhibit the mitogenic effect of oestrogen in these cells (40). In contrast, in a yeast expression system this variant ER does not have transcriptional or dominant negative activity (63).

Again the data available in the literature concerning the potential activity of the exon 3-deleted ER suggest that variant activity as well as wild-type ER activity can depend on the gene promoter used and the cellular milieu. Interestingly, reported in abstract form is the observation that the level of the exon 3-deleted ER transcript is higher in normal mammary epithelia compared to breast tumours and tumour cell lines (40). This raises the interesting possibility that the expression of this variant may decrease with breast tumourigenesis and the exon 3-deleted ER may have an important role in the control of ER signalling and the control of breast epithelial cell growth. In a range of 100 breast tumours, using an approach that allowed the investigation of the relative expression of multiple ER-deleted mRNAs, the exon 3-deleted variant transcript was low to undetectable (20), while another study suggested that the level of this variant was similar in all ER+ breast tumours and was therefore unlikely to be involved in the evolution of the ER+/PgR− breast cancer phenotype in contrast to the exon 7-deleted variant (63). However, no compari-

son with normal human breast tissue was made in either of these two latter studies.

### 5. Exon 2-deleted ER

An ER-like transcript deleted in exon 2 sequences was first demonstrated in T-47D human breast cancer cells (11). Subsequently, it was identified in MCF-7 cells (19) and both normal (16–18) and neoplastic breast tissues (16–18). The exon 2-deleted transcript could encode a truncated protein of approximately 16 kDa missing the entire DNA- and ligand-binding domains (Fig. 1, Table 1). The protein would only encode the A/B region of the wild-type ER up to amino acid 151 with an additional novel amino acid residue. The protein encoded by this transcript displayed no transcriptional activity of its own, but exhibited a mild dominant negative activity when over-expressed at least 20-fold relative to the wild-type ER protein (11). This transcript was found to be over-expressed in a tamoxifen-resistant MCF-7 cell line compared to the parent MCF-7 cells, although other ER variant transcripts were also differentially expressed in these two cell lines (19). Although such data support a role for altered ER variant expression in hormone independence, the mechanism or mechanisms by which this is achieved is unknown.

### 6. Multiple-exon-deleted and Other Deleted ERs

Several multiple-exon-deleted ER transcripts have recently been identified in human breast cancer cells (19), and in both normal and neoplastic human breast tissues (16–18, 20). These include both double- and triple-exon deletions. Deletions of exons 4 and 7 from the one transcript have been described in human breast cancer cells (19) and human breast tissue (18). Furthermore, this transcript is frequently expressed at a relatively high level in a wide range of human breast tumours (20). An exon 4- and 7-deleted ER transcript is predicted to encode a protein of approximately 39 kDa (18) deleted in the hinge region, lacking a nuclear localization signal and significant portions of the ligand-binding and AF-2 domains. No studies reporting putative function have been published. Leygue et al. (16, 20) have identified transcripts deleted in exons 2 and 3, and transcripts deleted in exons 3 and 4 in human breast tissues. This latter transcript was also identified in human breast tissues by Gotteland et al. (17). The transcript is predicted to encode an inframe protein of approximately 49 kDa, lacking ER amino acid residues encoded on exons 3 and 4, i.e. amino acids 216–365 (Fig. 1, Table 1). This protein would be unable to bind to DNA, would be missing a nuclear localization signal and part of the hormone-binding domain. Interestingly, the relative expression of this transcript is increased markedly in human breast cancer cells that have become oestrogen independent (A. Coutts, E. Leygue and L. Murphy, unpublished observation). No data are available with regard to the potential function of this protein in human breast cells, although the protein encoded by this transcript has no transcriptional or dominant negative activity in a rat aortic smooth muscle cell line model (61).

Triple-exon-deleted ER transcripts have been observed in MCF-7 human breast cancer cells (18) and in human breast cancer tissue (20). MCF-7 cells contain ER transcripts deleted in exons 3, 4 and 5 as well as transcripts deleted in exons 5, 6 and 7 (18). Leygue et al. (20) recently identified in human breast cancer tissues ER transcripts deleted in exons 2, 3 and 4 and exons 2, 3 and 7. No functional studies have been carried out on the proteins encoded by such transcripts; however both these transcripts were frequently detected at a relatively high level of expression in a wide range of human breast tumours (20). In addition, the detection of the exon 2, 3 and 4-deleted transcript was significantly correlated with high-grade tumours (20).

Deletions that are not exact exon deletions have also been described. Grahame et al. (21) identified in T-47D cells an ER-like transcript deleted of 462 bases from within exon 4 to within exon 5. This predicts for a putative protein containing 442 amino acids with an in-frame deletion of 153 amino acids of the wild-type ER protein (Fig. 1, Table 1). The predicted protein is deleted from the end of the DNA-binding domain to mid-ligand-binding domain. This same group observed an ER transcript deleted in a G residue (nucleotide 1463) (numbered according to (64) of wild-type ER sequence) at amino acid residue 411 in the hormone-binding domain of the ER. This resulted in a frame shift so that a truncated protein is encoded (Table 2). The predicted protein is identical to the wild-type ER up to amino acid residue 410, followed by seven novel amino acids. The protein would have an intact DNA-binding domain and hinge region but would be truncated in the ligand-binding domain. Similarly, Karnik et al. (22) identified an ER-like transcript in a tamoxifen-resistant metastatic human breast tumour that was deleted in a T residue in exon 6. This would generate a frame shift resulting in a protein identical to the wild-type ER up to amino acid residue 433 followed by five novel amino acids (Table 2). This protein is probably defective in its ligand-binding and AF-2 activities. Daffada and Dowsett (23) described a novel splice variant of the ER mRNA in normal human endometrial tissue and breast cancers. This variant consists of a deletion within exon 4 sequences to within exon 7 sequences. This variant is out of frame, is identical to the ER up to amino acid residue 277 and thereafter encodes another 32 novel amino acids (Fig. 1, Table 1). The predicted protein would lack a large part of the ligand-binding domain and the AF-2 domain, but would contain the AF-1 domain, the DNA-binding domain and the nuclear localization signal of the wild-type ER. Leygue et al. (20) have identified ER transcripts in a wide range of human breast cancer samples, which are deleted from within exon 3 to within exon 7. This transcript was frequently detected in breast tumours, and in particular its expression was significantly correlated to tumours with very high levels of wild-type ER up to amino acid residue 232 and would then encode a further 18 novel amino acids (Fig. 1, Table 1). However, the protein lacks some of the

DNA-binding domain, all of the ligand-binding domain, and the AF-2 function.

### 7. Truncated ER mRNAs

The truncated ER-like transcripts (24, 25), which consist of various combinations of exons 1, 2 and 3 of the normal ER mRNA followed by sequences that are not found in the wild-type ER mRNA, were initially identified on Northern blots as abundantly expressed smaller-sized ER transcripts in some human breast cancer biopsy samples. This analysis identified them as abundant or more abundant than the wild-type transcript in some human breast cancer samples (24). Subsequently, several of the cognate cDNAs for these truncated transcripts were cloned and characterized, and found to contain authentic polyadenylation signals and poly A tails. The clone 24- and clone 5-truncated transcripts were found in only one breast tumour but the clone 4-truncated ER mRNA was found to be expressed in a wide range of breast tumours (25). Clone 5, however, consisted of exon 1 and 3 followed by ER unrelated sequences, and therefore is an example of a mixed exon-deleted and truncated transcript. Clone 4 consists of exons 1 and 2 of the wild-type ER mRNA followed by LINE-1 sequences (25). It could encode a protein of approximately 24 kDa, which would be identical to amino acid residues 1–214 of the wild-type human ER protein (25) (Fig. 1, Table 1) and thereafter encodes another six novel amino acids that are not found in the wild-type human ER. If the clone 4 mRNA were translated it would encode a protein that is identical to the A/B region and the first 'zinc finger' of the normal ER protein, but would be missing the second 'zinc finger', nuclear localization domains and the E domain of the normal ER protein (4). However, the protein had no transcriptional or dominant negative activity in transient transfection assays (25). Support for a role for this variant in human breast cancer progression comes from data that show that the relative level of expression of this variant is significantly elevated in breast tumours versus normal mammary gland (39) and that the relative level of expression of this variant is significantly elevated in breast tumours with characteristics of poor prognosis and endocrine resistance versus those with characteristics of good prognosis and endocrine sensitivity (41).

### 8. Point Mutations in the ER

Several point mutations have been identified in the human ER. The first of them was a G-to-C mutation (30–32), which was a silent polymorphism at nucleotide 261 (using the numbering presented in (64)). Although this is a silent polymorphism, the B-region variant allele (B′) of the ER has been correlated with decreased levels of oestrogen binding in human breast cancers (65), increased history of spontaneous abortion in women with ER-positive breast cancer (66), increased height in women (67) and possibly increased prevalence of hypertension (68).

A C-to-T transition at codon 157 in exon 2 of the human ER appears to be the cause of oestrogen resistance in a man (36). The mutation results in a premature stop codon so that a protein truncated within exon 2 would be formed, encoding only the A/B region and missing both zinc fingers of the DNA-binding domain as well as the entire hormone-binding domain. This is the first identified disease causing mutation in the human ER. Interestingly, this study demonstrated that disruption of the ER gene need not be lethal in humans and identified the importance of oestrogen in bone maturation and mineralization in men as well as women (36).

Point mutations have been identified in the ER in some breast cancers. A silent polymorphism (T-to-C) at serine 10 has been identified by at least two independent groups (25, 29). A leucine to proline substitution at amino acid residue 296 has been identified in two breast tumours (33); however, the functional significance of this is unknown. A C-to-G change that is a silent polymorphism at proline 325 (33) has also been observed. Karnik et al. (22) identified an A-to-T nucleotide change in one breast tumour, which would alter Glu 352 to Val as well as several silent polymorphisms (C-to-T in Gly 276; G-to-T in Lys 472; C-to-G at Ala 505; T-to-C at His 577). However, none of these was frequently observed and none correlated with tamoxifen sensitivity or resistance in this group of human breast tumours. The point mutation changing Gly 400 to Val that was introduced into the human ER cDNA, as a cloning artifact, was shown to alter the receptor's affinity for oestrogen under certain conditions (69), as well as to enhance the oestrogenic activity of 4-hydroxytamoxifen in stable ER transfectants of MDA-MB-231 human breast cancer cells (64). Moreover, the ER from an MCF-7 tumour line, which was stimulated by tamoxifen, contains a point mutation so that Asp 351 was changed to a Tyr residue (34). This mutant ER was subsequently shown to result in increased oestrogenicity of a tamoxifen analog (35).

### 9. Insertions in the ER

ER mRNAs containing inserted sequences have been identified in approximately 9% of human breast tumours (26). Three types of inserted sequences were identified: one in which a complete duplication of exon 6 was found, one in which a complete duplication of exons 3 and 4 was found, and one in which 69 novel nucleotides had been inserted between the exon 5 and 6 sequences of the normal ER mRNA. The functional significance of such alterations is as yet unclear. However, the exon 6-duplicated ER-like mRNA predicts a protein of 51 kDa identical to the wild-type ER but would be truncated in the mid-E domain. Deletion and site-directed mutagenesis data suggest that such a protein would not bind oestradiol (2, 4, 71–74). Further, an important dimerization interface and the ligand-dependent AF-2 activity would be missing in the protein predicted from the exon 6-duplicated ER-like mRNA. However, a weaker constitutively active dimerization domain present in the DNA-binding domain, as well as the constitutive nuclear localization signal present in

exon 4 of the wild-type ER (75) and the ligand-independent AF-1 activity in the A/B domain would still be present (5). Preliminary data suggest that the protein encoded by this transcript has no ability to bind oestradiol and has little, if any, transcriptional activity using a classical ERE reporter gene construct (D. Douglas and L. Murphy, unpublished observations).

The predicted protein from the exon 3 and 4-duplicated ER transcript is around 82 kDa (Fig. 2, Table 1). It is identical to the wild-type ER protein up to amino acid residue 366, followed by another 380 amino acid residues encoded by exons 3 to 8. Therefore the amino acid residues encoded by exons 3 and 4 are completely duplicated. This protein would contain the AF-1 domain located in the A/B region of the wild-type ER, as well as the DNA-binding and dimerization domains and the constitutive nuclear localization signal of the wild-type ER protein, but would then have a third zinc finger encoded by exon 3, another nuclear localization signal followed by the normal E-domain containing ligand binding, AF-2 and dimerization functions. The presence of the extra ER residues from exons 3 and 4 would probably result in an altered structure of the protein, which may affect several of its normal functions. Preliminary data suggest that the protein encoded by this transcript has reduced oestradiol-binding activity and reduced, but still detectable, ligand-activated transcriptional activity (D. Douglas and L. Murphy, unpublished observations).

The unique 69-bp insertion is in-frame and codes for 23 novel amino acids inserted between residues 412 and 413 of the normal ER protein (Fig. 2, Table 1). This would result in a protein of approximately 69 kDa. While all residues of the wild-type ER are present in this protein the inserted sequence may cause an alteration of the structure in the E domain of this protein, so that some alteration or disruption of function may occur. Preliminary data suggest the protein encoded by this transcript has reduced oestradiol-binding activity and little, if any, transcriptional activity of its own (D. Douglas and L. Murphy, unpublished observations).

Interestingly, the identification of an immunoreactive ER-like protein of 80 kDa was recently reported in an MCF-7 subclone, which was oestrogen independent with respect to growth (45). The transcript possibly corresponding to this protein appeared to contain a precise duplication of both exons 6 and 7 (Table 1, Fig. 2). Also, an abnormal ER-like transcript was cloned from T-47$D_{CO}$ cells, which contained an insertion of approximately 130 nucleotides into exon 5 sequences (21). The inserted sequences displayed sequence similarity to the human *alu* family of repetitive sequences (21). The same group identified another mutant ER transcript in T-47$D_{CO}$ cells, in which two T residues were inserted in exon 3 resulting in a frame shift, changing amino acid 250 from methionine to an isoleucine, followed by a stop codon (21). The predicted protein would be truncated just beyond the last cysteine of the second zinc finger, with no hinge or ligand-binding domains (Table 2). Although no DNA binding/gel retardation analysis for this predicted protein was observed, the protein displayed weak constitutive transcriptional activity, and higher concentrations had weak inhibitory activity when expressed together with the wild-type ER (76). In addition, some small insertions (1-3 nucleotides) have been described in the ER mRNA of some breast cancer biopsy samples (22, 29, 33) (Table 2). The frequency and significance of these are not known.

## Conclusions and Unanswered Questions

There is a large amount of molecular evidence supporting the existence of variant and mutant ER proteins. While this evidence is derived mainly from characterization of mRNA species, data are now accumulating to suggest that the stable translation of ER variant mRNAs occurs at least in some human breast cancer tissues. This, in turn, suggests that any future examination of ER signal transduction and/or measurement of ER protein must take into account variant ER expression. The possible functions of variant ER proteins, either physiological or pathological, remain unclear, although correlative studies tend to support a role or roles for some ER variants in breast tumourigenesis and breast cancer progression. However, future speculation concerning these issues must take into account the presence of multiple ER variants in any one breast tissue sample, as well as the relative expression of each variant with respect to others, which can be altered in different groups of breast tumours, as discussed above. Furthermore, there are data that support the possibility that the pattern of ER variant expression can differ amongst different normal oestrogen target tissues (23), suggesting a possible role in the tissue-specific differences of ER signal transduction. These differences also dictate that analysis of putative function of any individual ER variant must also consider the cellular context as well as the promoter used to assess transcriptional function. This becomes increasingly important in the light of recent studies where novel oestrogen-responsive DNA sequences have been characterized, which remain quite distinct in structure–function activity and presumably mechanism from that classically determined using ERE sequences from the vitellogenin promoter (77–80). The recent cloning of a new ER, ER-beta (81, 82), with an overlapping but distinct pattern of tissue expression to the classical ER-alpha, also begs the question of whether the two ERs can interact and how the variant receptor forms may affect either or both signal transduction pathways.

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# APPENDIX 10

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# THE PATHOPHYSIOLOGICAL ROLE OF ESTROGEN RECEPTOR VARIANTS IN HUMAN BREAST CANCER.

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**Summary.**

The accumulated evidence supports the expression of estrogen receptor variants at both the mRNA and protein levels. The relative level of expression of some estrogen receptor variant mRNAs and possibly progesterone receptor variant mRNAs is altered during breast tumorigenesis and breast cancer progression. The altered expression of estrogen receptor variants may effect estrogen signal transduction as well as the interpretation of assays where the estimation of estrogen receptor levels is used as a guide to treatment strategies and prognosis .

**Introduction.**

The estrogen receptor is considered pivotal in the mechanism by which estrogen interacts with its target cells and mediates its specific effects. Classically, the estrogen receptor is considered to be a ligand activated transcription factor, which upon estrogen binding undergoes conformational changes which allow it to dimerize, tightly bind to estrogen responsive DNA sequences and alter transcription of target genes [1, 2]. However, the diverse effects of estrogen on target tissues [3-6] and the observation that many human breast cancers develop estrogen independence despite the continued expression of the estrogen receptor [7] suggest that the concept of estrogen action described above is unlikely to simply explain all aspects of estrogen action [2, 8]. Evidence has accumulated over the last decade supporting the existence of estrogen receptor variants [9, 10]. Therefore the possibility exists that estrogen receptor variants may have a pathophysiological role in estrogen action. For example, the pattern of estrogen receptor variant expression may influence which set of estrogen responsive genes are transcribed. The following discussion reviews the evidence available to support a pathophysiological role of estrogen receptor variants in human breast tissues.

**Structure of Estrogen Receptor Variant mRNAs.**

Most data supporting the existence of estrogen receptor (ER) variants have been at the mRNA level. Two main structural patterns of estrogen receptor variant mRNAs have been consistently identified: the truncated ER mRNAs [11] and the deleted ER mRNAs [12]. Using a long range reverse transcription-polymerase chain reaction analysis (RT-PCR) [13] which detects all deleted ER variant mRNAs at a frequency relative to their initial mRNA representation in the unamplified sample [14], the most frequently detected and likely the most abundant deleted ER variant mRNAs in human breast tumors appear to be the

exon 7 deleted ER mRNA [13, 15] and the exon 4 deleted ER mRNA [13, 16]. However, in some breast tumors other deletion variants such as an exon 3+4 deleted ER mRNA [13] and an exon 4+7 deleted ER mRNA [13] have been frequently detected. An exon 5 deleted ER variant mRNA is rarely detected using the long range RT-PCR approach, suggesting that its abundance is low compared to several other ER deleted mRNAs. However, when specific PCR primers are used to measure only the exon 5 deleted ER mRNA relative to the wild-type ER mRNA, the levels of the exon 5 variant mRNA are found to vary amongst breast tumor samples [17] as well as between normal breast tissues and breast tumors [18]. Also truncated ER variant mRNAs have been frequently detected at relatively high abundance in several human breast tumors [19, 20]. In fact the truncated ER variant mRNAs are the only ER variant transcripts that have been detected by Northern blotting analysis and the entire cDNAs cloned and sequenced [11, 19]. A commonly expressed truncated ER mRNA is the clone 4 truncated ER mRNA. The predicted proteins of these relatively most abundant ER variant transcripts are shown schematically in Figure 1. All of these variant transcripts will encode ER proteins missing some structural/functional domains of the wild-type ER. In many cases i.e. the exon 7 deleted, the exon 4+7 deleted and the clone 4 truncated ER variant mRNAs will encode C-terminally truncated proteins in which the ligand binding domain, the ligand dependent transcriptional activity (AF-2) and the ligand dependent dimerization domain are significantly impaired. Although the exon 4 deleted and the exon 3+4 deleted transcripts are inframe, studies indicate that the proteins encoded by these transcripts cannot bind ligand and have little, if any, ligand dependent transcriptional activity [16, 21]. Furthermore, the proteins encoded by the clone 4 truncated, the exon 3 deleted, the exon 3+4 deleted and the exon 4+7 deleted variant mRNAs are all unlikely to bind significantly to classical estrogen responsive DNA sequences. A common feature of all the relatively abundantly expressed ER variant mRNAs described above, is they would encode proteins with intact A/B regions. This region has been described to contain a promoter and cell-type specific transcriptional activity [22, 23], although its ability to function in the complete absence of an intact DNA binding domain is unexplored. In summary, a large body of molecular data exists to support the potential expression of ER variant proteins.

**Specificity of Estrogen Receptor Variant Expression.**

The available studies provide evidence for an extensive and complex pattern of alternative splicing associated with the estrogen receptor gene, which appears to be altered during breast tumorigenesis. It has been suggested that the complex pattern of exon deleted ER variant mRNA expression is specific for the estrogen receptor, since similar variants for the glucocorticoid receptor and the retinoic acid receptors alpha and gamma have not been found in breast tumor tissues [24]. We have also investigated the pattern of exon deleted variant mRNA expression in breast tumors using a long range RT-PCR approach [13] for the progesterone receptor (PR) [25], the glucocorticoid receptor (GR) [26] and the vitamin D3 receptor (VR) [27]. Our data, shown in Figure 2, demonstrate that little, if any, deleted variant mRNAs for the GR and VR were detected in the three breast tumors examined. However, in these same breast tumors, several exon deleted variant mRNA species for both the ER and PR were abundantly expressed (Figure 2). Importantly, our published data have shown that the expression of the exon 5 deleted ER variant mRNA and the truncated clone 4 ER variant mRNA is elevated in breast tumors compared to normal breast tissues [18, 28] and our preliminary data suggest that the level of the exon 6 deleted PR variant mRNA was more highly expressed relative to the wild-type PR mRNAs in breast tumors compared to normal breast tissues (Leygue, Dotzlaw, Watson, Murphy, unpublished data). These data suggest that the mechanisms generating alternatively spliced forms of both ER and PR are unlikely to be due to a generalized deregulation and/or alteration of splicing processes within breast tumors. They also suggest that the mechanism(s) is specific for the sex steroid hormone receptor genes and the alterations seen in breast tumors may have a role in altered actions of estrogens and progestins in human breast tumorigenesis.

**Identification of Estrogen Receptor Variant Proteins.**

Recent data published from several independent groups strongly support the detection of estrogen receptor like proteins which correspond to some of the previously identified estrogen receptor variant mRNAs in both cell lines and tissues *in vivo*.

An ER-like protein consistent with that predicted to be encoded by the exon 5 deleted ER transcript is expressed naturally in some BT 20 human breast cancer cell lines [29]. A monoclonal antibody specific to the predicted unique C-terminal amino acids of an exon 5 deleted ER protein was developed, and used to demonstrate immunohistochemically the presence of this protein in

several human breast cancer samples [30] . An exon 4 deleted ER variant mRNA has been identified in both normal and neoplastic ovarian tissue [31] Western blotting analyses revealed the presence of the expected 65 kDa wild-type ER protein as well as a 53 kDa protein which was recognized by ER antibodies to epitopes in the N-terminus and C-terminus of the wild-type protein, but not with an antibody recognizing an epitope encoded by exon 4 [31]. These data strongly support the hypothesis that the small sized ER-like protein in the ovarian tissue extracts was encoded by an exon 4 deleted ER variant mRNA.

Recently, we have analyzed several human breast tumors immunohistochemically for ER expression, comparing side-by-side an antibody recognizing an N-terminally localized epitope in the wild-type ER protein (1D5), and an antibody recognizing a C-terminally localized epitope in the wild-type ER protein (AER311). It was found that although in many tumors the immunohistochemical results using each antibody showed good concordance with each other, in some tumors the results were discordant, with the signal tending to be higher with the N-terminal antibody. Since many of the proteins predicted from variant ER mRNAs would be truncated at the C-terminus and not contain the epitope recognized by the C-terminal antibody, one interpretation of these data would be that truncated variant ER proteins are more highly expressed in the discordant group of tumors [32]. This hypothesis was tested by investigating the pattern and relative expression of variant ER mRNAs in the discordant and concordant groups of breast tumors. Several ER variant mRNAs (the clone 4 truncated ER mRNA, the exon 2, 3 plus 7 deleted ER mRNA, the exon 2, 3 plus 4 deleted ER mRNA and the variant deleted within exon 3 to within exon 7) which encode putative truncated ER-like proteins that would be recognized only by an N-terminally targeted antibody were preferentially and more highly expressed in the discordant breast tumor group [33]. While this indirect approach does not specifically identify ER variant proteins, the data suggest that the ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER measured by immunodetection assays using N- or C-terminal antibodies.

The accumulated data provide strong support for the ability of, at least some ER variant mRNAs to be stably translated into proteins detectable by conventional methodologies. Such proteins may have a functional role in altered estrogen signal transduction in human breast tumorigenesis.

**Functional Significance of Estrogen Receptor Variant Expression.**

The important question to be addressed now is the role of ER variants in those tissues in which they are expressed. One approach has been to recombinantly express individual ER variants, alone and together with the wild-type ER, and determine effects on transcriptional activity as measured by a classical estrogen response element (ERE)-reporter gene. This has demonstrated the exon 5 deleted ER to have constitutive activity [34] and wild-type ER inhibitory activity [30]. As well the exon 3 deleted ER [35], the exon 4 deleted ER [31] and the exon 7 deleted ER [15] have been identified in some sytems to have wild-type ER inhibitory activity. Some ER variants such as the exon 3+4 deleted variant have been shown to enhance wild-type ER activity, at least at the basal level [36]. Other ER variants such as the truncated clone 4 ER have no detectable activity in similar assays [11].

Another approach has been to determine if the ER variants are differentially expressed in normal versus neoplastic breast tissues. The accumulated data support the increased expression of the exon 5 deleted [18, 31] and the clone 4 truncated ER mRNAs [28] in breast tumors with good prognositic features compared to normal breast tissues. Furthermore it has been suggested that decreased expression of an exon 3 deleted ER mRNA occurs in breast tumors compared to normal breast tissue [37]. Further changes seem to occur during the later stages of breast cancer progression since the relative level of expression of the clone 4 truncated ER mRNA was found significantly elevated in breast tumors with characteristics of poor prognosis and endocrine resistance versus those with characteristics of good prognosis and endocrine sensitivity [20]. Moreover, increased expression of an exon 3+4 deleted ER was found associated with the estrogen independent, ER positive phenotype in a breast cancer cell line model [36] and preliminary data support the functional involvement of the overexpressed ER variant in the estrogen independent phenotype (Coutts, Leygue, Murphy, unpublished data).

Inconsistent results have been obtained with respect to stably overexpressing an individual ER variant in a hormone dependent breast cancer cell line and the resulting development of endocrine resistance [10, 38]. The data suggest that altered ER variant expression may contribute to altered estrogen receptor activity which together with other factors will contribute to breast cancer progression and the eventual development of hormone independence and resistance endocrine therapy [7].

## Conclusions.

Estrogen receptor variants can be detected at both the mRNA and protein levels. The level of expression of some ER variants is altered during breast tumorigenesis and breast cancer progression. The functional involvement of ER variants and possibly PR variants in breast cancer progression and altered responses to these steroid hormones requires further detailed investigation. More immediately, the impact of expression of variant ER and PR on the determination of ER and PR immunohistochemically as markers of prognosis and treatment response in breast cancer requires assessment.

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## LEGENDS TO FIGURES

### Figure 1

Schematic diagram of the wild-type human estrogen receptor (ER) cDNA, which contains 8 different exons coding for a protein divided into structural and functional domains (A-F).
Region A/B of the receptor is implicated in trans-activating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another trans-activating function (AF-2). The numbering on the top of the cDNA refers to the nucleotide position as defined in reference [39] . Below the wild-type ER cDNA are the various putative exon deleted and truncated ER cDNAs. ATG shows the translation initiation codons, TGA shows the inframe translation termination codons and the numbering below the cDNA refers to the amino acid positions as defined in reference [39]. D = deletion, and the estimated molecular mass (kDa = kiloDaltons) of each open reading frame is shown in brackets. Molecular masses were estimated using MacVector version 4.1.4 software.

### Figure 2

Autoradiograph of long range RT-PCR analysis for ER, PR, GR and VR mRNA isolated from three human breast tumor biopsy samples. All tumors were ER+ and PR+ by ligand binding assay. The primers and RT-PCR conditions for ER and PR were as previously described [13, 25] except that the annealing time was 30 seconds only. The primers for the GR are as previously described [24] and located in exons 2 and 8, respectively. The RT-PCR conditions were those described above for ER and PR. The primers for the VR are: VR-U 5'- GAAGCGGAAGGCACTAT-3', sense 155-171 as defined in [27] ; VR-L 5'-GAGCACAAGGGGCGTTA -3', antisense 1240-1256 as defined in [27]. D = deletion. The numbers beside the arrows represent the sizes (bp) of the PCR product: 1381 is WT-ER; 1197 is D7-ER; 1100 is WT-VR; 1093 is WT-PR; 1045 is D4-ER; 987 is WT-GR; 962 is D6-PR; 928 is D3+4-ER; 861 is D4+7 ER; 787 is D4-PR; 580 is D3+7-ER [13, 25].

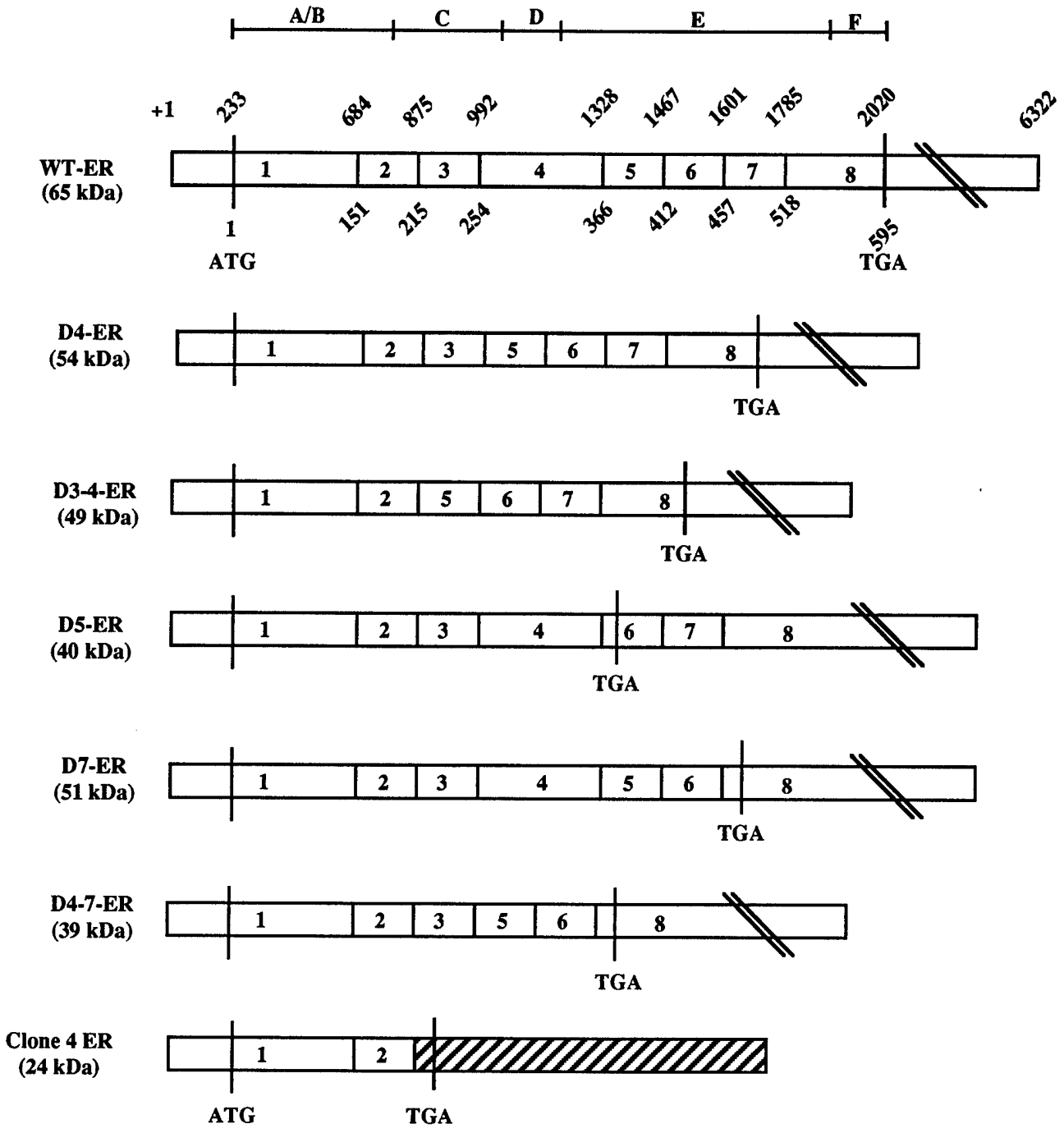


**Figure 1 Murphy et al.,**

Figure 2 Murphy *et al.*,